(19) Canadian Intellectual Property Office
An Agency of Industry Canada

Office de la Propri,t, Intellectuelle du Canada
Un organisme d'Industrie Canada

(11) CA 2 290 722 (13) A1

(40) 08.06.2001
(43) 08.06.2001

(12)

(21) 2 290 722

(22) 08.12.1999

(51) Int. Cl.[7]: C07K 14/47, A61P 31/00, A61K 38/17, C07K 16/18, A61K 39/385, G01N 33/53, G01N 33/566

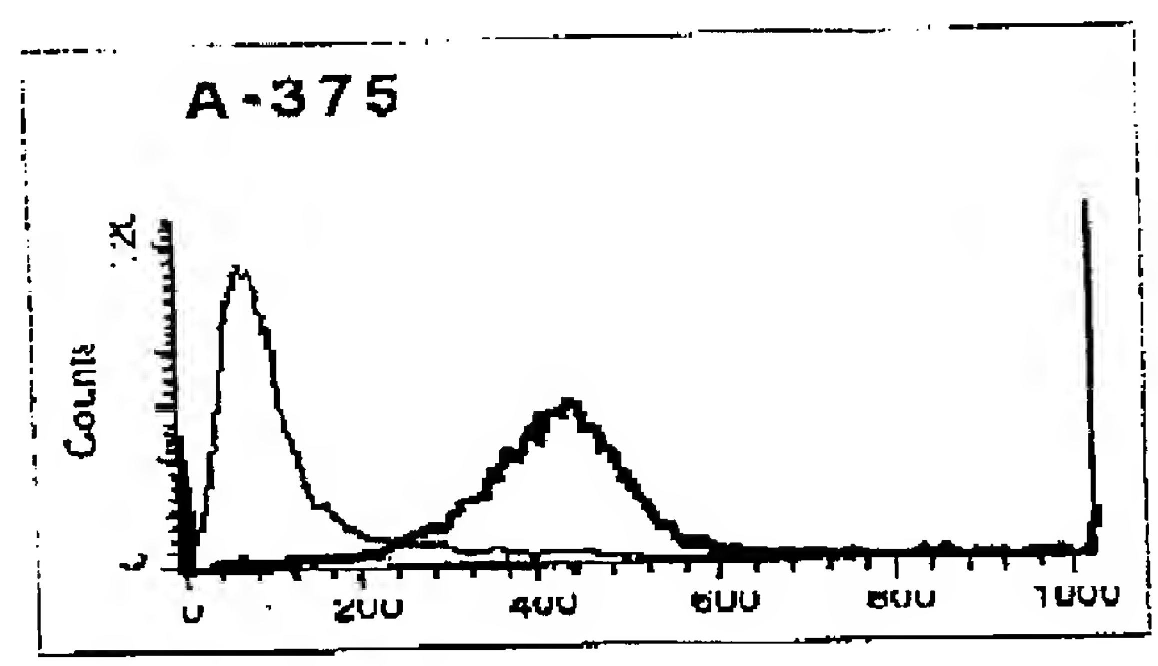
(71)
NOVOPHARM BIOTECH INC.,
147 Hamelin Street, WINNIPEG, A1 (CA).

(72)
MAITI, PRADIP K. (CA).
HERMAN, WILLIAM (CA).
DAN, MICHAEL D. (CA).
KAPLAN, HOWARD A. (CA).
MACDONALD, GLEN C. (CA).
ENTWISTLE, JOCELYN M. (CA).
LEWIS, KEITH E. (CA).
FAST, DARREN G. (CA).

(74)
RIDOUT & MAYBEE

(54) PRODUITS CONTENANT UNE SEQUENCE PEPTIDIQUE CONSENSUS
(54) CONSENSUS PEPTIDE PRESENTING ENTITIES

(57)
The invention provides antigen-binding-fragments specific for tumor cells and effective in treatment and/or diagnosing tumors. Methods of use are also provided as are methods for screening for additional such antigen-binding-fragments and the products obtained thereby.



OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

(12) (19) (CA) **Demande-Application**

(21) (A1) **2,290,722**

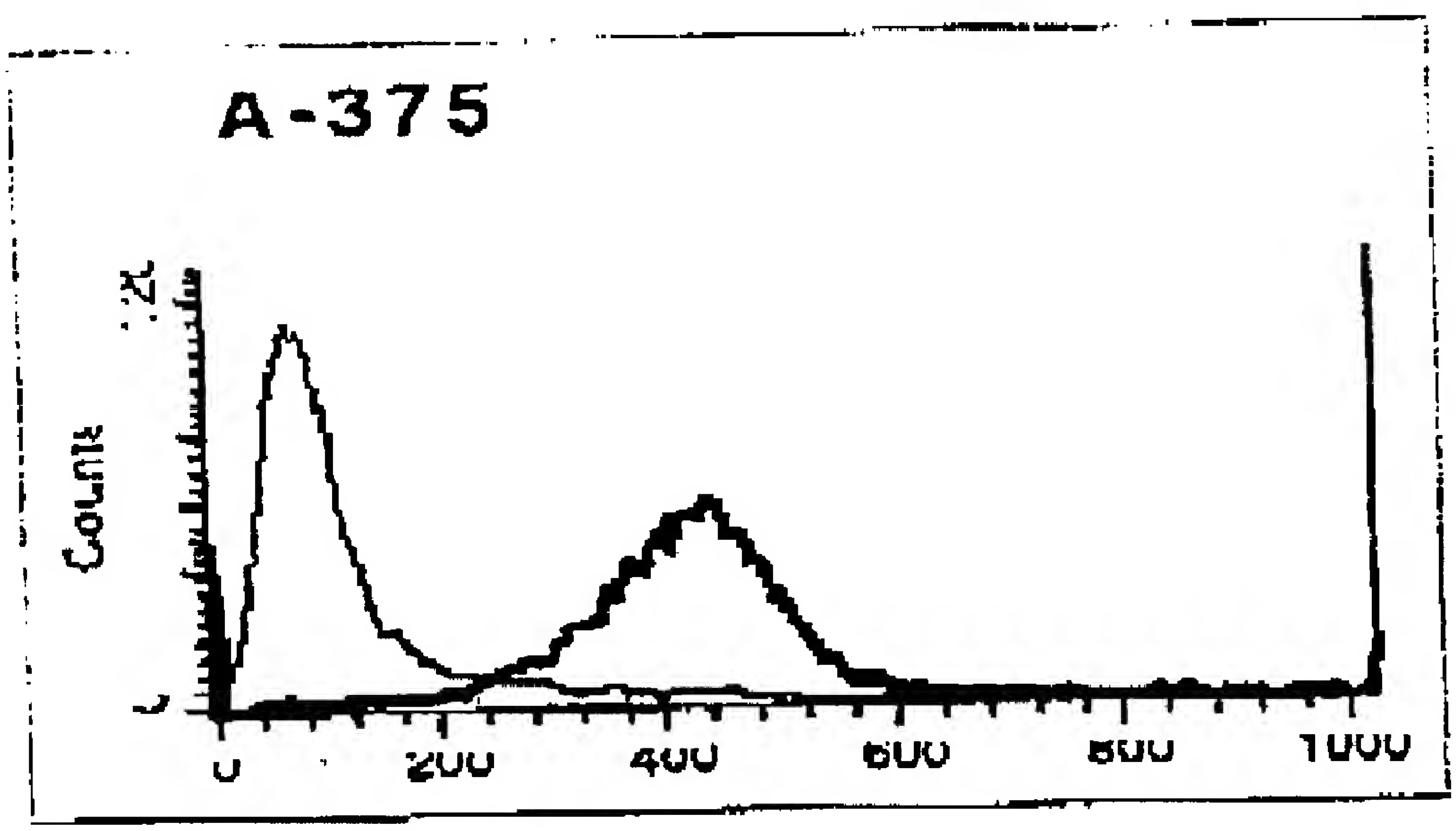
(22) 1999/12/08
(43) 2001/06/08

(72) MACDONALD, GLEN C., CA
(72) KAPLAN, HOWARD A., CA
(72) MAITI, PRADIP K., CA
(72) ENTWISTLE, JOCELYN M., CA
(72) LEWIS, KEITH E., CA
(72) FAST, DARREN G., CA
(72) DAN, MICHAEL D., CA
(72) HERMAN, WILLIAM, CA
(71) NOVOPHARM BIOTECH INC., CA

(51) Int.Cl.[7] C07K 14/47, G01N 33/566, G01N 33/53, A61K 39/385, C07K 16/18, A61K 38/17, A61P 31/00

(54) **PRODUITS CONTENANT UNE SEQUENCE PEPTIDIQUE CONSENSUS**

(54) **CONSENSUS PEPTIDE PRESENTING ENTITIES**



(57) The invention provides antigen-binding-fragments specific for tumor cells and effective in treatment and/or diagnosing tumors. Methods of use are also provided as are methods for screening for additional such antigen-binding-fragments and the products obtained thereby.

316083002521

Abstract of the Disclosure

The invention provides antigen-binding-fragments specific for tumor cells and effective in treatment and/or diagnosing tumors. Methods of use are also provided as are methods for screening for additional such antigen-binding-fragments and the products obtained thereby.

CONSENSUS PEPTIDE PRESENTING ENTITIES

I. CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

II. STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

Not applicable.

III. TECHNICAL FIELD

The invention relates to antigen-binding fragments that are specific for individual or families of PPEs (PPEs), including stress protein-peptide complexes associated with tumors (SPPCs), particularly human tumors, and compositions thereof. The compositions are suitable for diagnostic, palliative and therapeutic use. The invention further provides methods of making and screening for the antigen-binding fragments. The invention further encompasses compositions containing peptide-presenting entities, which share antigenic determinants with tumor-associated SPPC (including derivatives thereof) and methods of use thereof. The tumor-specific SPPC are particularly useful in generating antigen-binding fragments specific for target tumors and in eliciting tumor-specific immunogenic responses.

V. BACKGROUND ART

A. Heat Shock Proteins

Heat shock proteins ("HSP"s) form a family of highly conserved proteins that are widely distributed throughout the plant and animal kingdoms. On the basis of their molecular weights, HSPs are grouped into six different families: small (hsp20-30 kD); hsp40; hsp60; hsp70; hsp90; and hsp100. Although HSPs were originally identified in cells subjected to heat stress, they have been found to be associated with many other forms of stress such as infections, and are thus more commonly known as "stress proteins" ("SP"s).

Members of the mammalian hsp90 family include cytosolic hsp90 (hsp83) and the endoplasmic reticulum counterparts hsp90 (hsp83), hsp87, Grp94 (ERp99) and gp96. See for instance, Gething et al. (1992) *Nature* 355:33-45. Members of the hsp70 family include cytosolic hsp70 (p73) and hsp 70 (p72), the endoplasmic reticulum counterpart BiP (Grp78), and the mitochondrial counterpart hsp 70 (Grp75). Members of the mammalian hsp60 family have only been identified in the mitochondria.

A variety of recent reports corroborate an association between the presence of SPs on the cell surface and cancer. The relationship between SPs and cancer was discovered in the course of efforts to identify cancer-associated antigens by their ability to elicit protective immunity to cancer challenges. The approach typically involved fractionating tumor homogenates into various protein components by conventional chromatographic methods and using these fractions to immunize animals just prior to challenge with live cancer cells. The fractions that elicited protection against the cancer were then repeatedly refractionated until apparently homogeneous preparations were obtained. To the surprise of investigators, all the well-characterized molecules identified by such methodology turned out to be SPs of the hsp90 or hsp70 family, even from cancers of diverse historic origins. See, Srivastava et al., Heat Shock Proteins Come of Age (supra). Naturally, this finding fostered a focused interest in using such fractions to immunize cancer patients against tumor tissue.

SPs are ubiquitous within cells, one of the roles of SPs is to chaperone peptides from one cellular compartment to another and to present the peptides to the major histocompatability complex (MHC) molecules for cell surface presentation to the immune system. In the case of diseased cells, SPs also chaperone viral or tumor-associated peptides to the cell surface. Li and Srivastava (1994) *Behring Inst Mitt* 94:37-47; and Suzue et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:13146-13151. The chaperone function is accomplished through the formation of complexes between the SPs and proteins and between SPs and viral or tumor-associated peptides in an ATP-dependent reaction. These complexes are termed "SPPC"s herein. SPs bind a wide spectrum of peptides in an ATP dependent manner. The bound peptides appear to be a random mix of peptides. The mixtures and exact natures of the peptides have not been determined. The association of SPs with various peptides has been observed in normal tissues as well and is not a tumor-specific phenomenon. See Srivastava (1994) *Experientia* 50:1054.

For instance, expression of hsp26, hsp60, hsp70 and hsp90 on the surface of human chronic myeloid leukemia (CML) cells from patients has been observed. Chant et al. (1995) *Br. J. Haematol.* 90:163-8. Cell surface expression of hsp70 has been detected on normal, premalignant and malignant human oral mucosa. Kaur et al. (1998) *Oral Oncol.* 34:93-8. A correlation of hsp70 expression with clinicopathological features showed a positive association with the severity of dysplasia in oral mucosal epithelium.

Ito et al. ((1998) *J. Oral. Pathol. Med.* 27:18-22), reported that they examined 24 specimens of squamous cell carcinoma of the tongue and found that, although SP immunohistochemistry revealed changes in expression during tumorigenesis of squamous epithelium of the tongue, there was no observed correlation with other clinical features studied (survival period, stage, lymph node metastasis, histological grade or p53 immunostaining).

It is currently believed that the antigenicity of SPPCs results not from the SP per se, but from the complex of peptide associated with the SP. This conclusion is based on a number of characteristics of the SPPCs. There are no differences in the structure of SPs derived from normal and tumor cells. Certain SPPCs lose their immunogenicity upon treatment with ATP. Udono et al. (1993) *J. Exp. Med.* 178:1391-1396. Such loss of immunogenicity is due to dissociation of the SPPC into its SP and peptide components.

Vaccination with a mixture of SPPCs can induce a potent immune response. In some instances, the immune response is humoral, as evidenced by the anti-peptide IgG response in BCG-primed mice injected with covalent hsp65 complex or hsp65-oligosaccharide complexes. Del Guidice *Experentia* (1994) 50:1061. In contrast, vaccination with mixtures of SPPCs from tumor cells appears to generate a strong cell mediated response with little more than a weak humoral response even after prolonged immunization. Srivastava et al. *Int. J. Cancer* (1984) 33:417-422. The explanation for the absence of a strong humoral response is that vaccination of tumor cell-derived SPPCs favors a Th1 immune response and would therefore, by definition, down-regulate any potential antibody response (Th2) directed against the tumor. Srivastava (1994) *Experentia* 50:1054-1060. This potential for dampening humoral immunity through SPPC vaccination, has led to its proposal as a method for suppressing allergic reactivity to different allergens. PCT/CA97/00897. Further, because the immune response, with respect to SPPC vaccination, is skewed in the direction of cell mediated immunity it has been suggested that humoral immunity plays but a minor role against neoplasia. (1998) *Biol. Chem.* 379:295.

In a therapeutic context, it has been proposed to use SP-antigen complexes as vaccines. In particular, U.S. Patent No. 5,750,119 to Srivastava discloses a multi-step, cancer patient-specific method for inhibiting the proliferation of a tumor in a mammal, by (a) removing tumor cells from the mammal; (b) isolating all SPPCs from the tumor cells; and (c) administering the isolated SPPCs back to the mammal in order to stimulate in the mammal a tumor-specific immune response. Hsp70-peptide, hsp90-peptide and gp96-peptide complexes are itemized as complexes having particular vaccine utility. Nevertheless, in the practice of the method disclosed by Srivastava, it is not considered necessary or even practical to isolate a specific peptide involved or even the particular SPPC involved in eliciting the immune response.

Moreover, Srivastava postulated that, "the prospect of identification of the immunogenic antigens of individual tumors from cancer patients (or even of 'only' several different types of immunogenic antigens in case the antigens are shared), is daunting to the extent of being impractical." On this basis, Srivastava proposes immunizing a mammal harboring a tumor with a mixture of SPPCs derived from the animal's own tumor, without isolating complexes specific to the tumor and without attempting to characterize complexes which are found on more than one tumor in one mammal.

U.S. Patent No. 5,837,251 discloses a method of eliciting an immune response in a mammal comprising administering a specified low dose of a SP peptide and an antigenic peptide. The antigenic peptide can be provided exogenously, that is, noncovalently reacted with the SP to form a complex, or it can be endogenous i.e. naturally occurring in a native complex. Again, the native material is a mixture of SPPCs and is not free of complex associated with normal cells. WO 99/22761 relates to conjugate peptides engineered to non-covalently bind to heat shock proteins. These peptides can be used to link antigenic peptides to heat shock proteins.

B. Anti-idiotypic Antibodies

The network hypothesis of Lindemann ((1973) *Ann. Immunol.* 124:171-184) and Jerne ((1974) *Ann. Immunol.* 125:373-389) offers an elegant approach to transform epitope structures into idiotypic determinants expressed on the surface of antibodies. According to the network concept, immunization with a given tumor-associated antigen will generate production of antibodies against this tumor-associated antigen, termed Ab1; this Ab1 is then used to generate a series of anti-idiotype antibodies against the Ab1, termed Ab2. Some of these Ab2 molecules

can effectively mimic the three-dimensional structure of the tumor-associated antigen identified by the Ab1. These particular anti-idiotypes called Ab2[illegible] fit into the paratopes of Ab1, and express the internal image of the tumor-associated antigen. The Ab2[illegible] can induce specific immune responses similar to those induced by the original tumor-associated antigen and can, therefore, be used as surrogate tumor-associated antigens. Immunization with Ab2[illegible] can lead to the generation of anti-anti-idiotype antibodies (Ab3) that recognize the corresponding original tumor-associated antigen identified by Ab1. Because of this Ab1-like reactivity, the Ab3 is also called Ab1' to indicate that it might differ in its other idiotopes from Ab1.

A potentially promising approach to cancer treatment is immunotherapy employing anti-idiotype antibodies. In this form of therapy, an antibody mimicking an epitope of a tumor-associated protein is administered in an effort to stimulate the patient's immune system against the tumor, via the tumor-associated protein. WO 91/11465 describes methods of stimulating an immune response in a human against malignant cells or an infectious agent using primate anti-idiotype antibodies. However, not all anti-idiotype antibodies can be used in therapeutic regimens against tumors. Moreover, since different cancers have widely varying molecular and clinical characteristics, it has been suggested that anti-idiotype therapy should be evaluated on a case by case basis, in terms of tumor origin and antigens express.

Anti-Id monoclonal antibodies structurally resembling tumor-associated antigens have been used as antigen substitutes in cancer patients. Herlyn et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:8055-8059; Mittleman et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:466-470; Chatterjee et al. (1993) *Ann. N.Y. Acad. Sci.* 690:376-278. It has been proposed that the anti-Id provides a partial analog of the tumor-associated antigen in an immunogenic context.

In contrast to the uncharacterized mixtures of SPPCs and artificially engineered SP-antigens previously described, we have now found that an antigen-binding fragment of an antibody that binds specifically to a tumor-associated SPPC is effective at eliciting a useful anti-tumor response. We have also found that this antibody, hereinafter referred to as "H11", recognizes tumor-associated SPPCs containing various different peptides. We have found that tumor-associated SPPCs contain peptides that conform to a consensus peptide motif ("consensus conforming peptides" or "CCPs"), to provide a family of tumor-associated SPPCs. Therefore, we have found that cancer-associated SPPCs share a consensus peptide motif, which is common to many tumors and that, peptides which conform to this consensus motif, can be used to

generate antigen-binding fragments, which bind to cancer-associated SPPCs, as well as, for making corresponding anti-Ids and for preparing compositions useful for eliciting an anti-tumorigenic response.

VI. SUMMARY OF THE INVENTION

In one aspect of the invention, we provide a method for identifying specific peptide presenting entities (PPEs) which are immunologically cross-reactive with tumor-associated SPPCs of at least one target tumor by:

1) identifying within a library of ligand-binding molecules, a subset consisting of one or more of such molecules, that binds to both:
   a) SPPCs found on the surface of target tumor cells; and
   b) one or more PPEs within a population of PPEs, said population containing a suitable representation of PPEs wherein the peptide portion corresponds to a consensus peptide motif described herein; and,
2) selecting PPEs bound to such fragments.

In some preferred embodiments of this aspect of the invention:

a) the consensus peptide motif is a hydrophobic motif comprising at least 3 hydrophobic amino acids within a span of 7 consecutive amino acids, preferably at least 2 such hydrophobic amino acids being non-contiguous, and more preferably 3;
b) the PPEs are SPPCs, preferably of the HSP70 and HSP90 families;
c) the population of PPEs comprises or is limited to a substantial representation of all potential consensus conforming peptides which correspond to each of the actual consensus conforming peptides found within proteins expressed in cells of each such target tumor;
d) the population of ligand-binding molecules is a large naïve library of antigen-binding fragments which can optionally be pooled with at least one additional library of fragments derived from a parental binding fragment which binds specifically to an SPPC (preferably containing SPs of both the HSP70 and HSP90 families); or
e) the population of ligand-binding molecules is derived from a parental binding fragment which binds specifically to an SPPC.

f) the population of ligand-binding molecules are single domain antibody fragments or loop structures as discussed below.

g) the population of antigen-binding fragments competes with H11 for binding to the target tumor

h) the consensus motif is designed to establish a strong likelyhood that peptides conforming to the motif would be found in association with MHC or HSP on the surface at least one (preferably a plurality and more preferably substantially all) cancers cell within a population of cancers cells of different individuals with cancer (preferably of the same cancer type), when a suitable number of such cells of each such individuals are tested.

Another aspect the invention is directed to a method of identifying a population of peptides containing a representation of peptides associated with SPPCs found specifically on the surface of cells of a target tumor by:

a) using a set of degenerate nucleic acid probes encoding permutations of a consensus peptide motif described herein (i.e. encoding proteins in which a peptide conforming to the motif is present) to identify those mRNA transcripts within cells of the target tumor that encode said motif; and,

b) manipulating the mRNA transcripts so identified to obtain the peptides.

In some preferred embodiments of this aspect of the invention:

Tumor cell-specific mRNA encoding specific consensus conforming sequences can be identified by any method known in the art. Preferably, an array of oligonucleotide probes is generated that encodes at least a subset of such consensus sequences. These probes are then used in a variety of ways to limit the number of peptides that are screened. In particular, cDNA complementary to the probes can be identified, cloned and expressed so that the peptides produced thereby can be subject to the PPE screening assay described herein. Alternatively, the probes are arrayed in a manner such as provided by Affymetrix® GeneChip technology so that probes of a single sequence are at identifiable spots on the chip. The technology is described for instance in US Patent Nos. 5,527,681; and 5,510,270. The chips are hybridized with mRNA or cDNA and the spots at which hybridization occurs are identified. The sequence for each spot is known and the peptide encoded thereby can be synthesized and subject to the screening assay described herein. With respect to tumor cells it is desirable to obtain a substantially complete

representation of consensus conforming sequences within the tumor cell using such probes in order to identify substantially all potential CCPs that could be brought to the surface of the tumor cell by an SP.

Methods of making the probes are known in the art. Any suitable DNA synthesizer can be used. As described in Example 13, approximately 4×10^6 different probes are initially obtained. The methods such as identifying cDNA complementary to the probes and DNA array screening of mRNA or cDNA narrow these peptides to a subset that represents proteins actually expressed.

In the case of cDNA screening, a cDNA library is first obtained, for instance from a cancer or tumor cell line. The single-stranded DNA is isolated. The probes are then annealed to the single-stranded DNA under stringent hybridization conditions. After an amount of time sufficient to anneal the probes to the cDNA, the single-stranded regions are digested to yield the double-stranded region. The remaining double-stranded regions are then ligated (singly or in concatenation) and transfected into a suitable expression vector. The cloned sequences can be sequenced. Expressed peptides can be isolated, optionally lengthened as described in Example 13, associated with SPs or other peptide presenting portions as described herein and subject to the screening assay to determine which peptides are found associated with SPs in a cancer-dependent manner.

In the case of oligonucleotide arrays, the probes are manufactured on the chip(s) and subject to stringent hybridization to mRNA or cDNA. Preferably the mRNA or cDNA are obtained from a cancer cell. More preferably, they are obtained from a human cancer cell. The spots to which the mRNA or cDNA anneal are then determined, for instance by the GeneChip method. As the sequence of each spot is known, the peptide encoded thereby can be synthesized, associated with SP and screened by the assays described herein. Optionally, the peptides are lengthened as described in Example 13 or in the course of preparing a set of consensus conforming peptides for screening in the form of PPEs i.e. by randomly adding one of each of the amino acid residues to each of consensus conforming peptides. Each amino acid that is added multiplies the starting population of consensus conforming peptides by a factor of 20, unless certain exclusion or inclusion criteria are applied as discussed below and in the art. For example, permutations of such added amino acids that do not exist within databases of human

proteins can be identified by searching for the occurrence of actual consensus conforming peptides within the literature and assessing the flanking amino acid residues.

In accordance with another aspect of the invention, we identify inclusion and exclusion criteria that can be applied to reduce the number of PPE candidates that are screened for immunological cross-reactivity with tumors associated SPPCs. It is to be understood that those criteria can be applied to one or more of stages of screening identified herein:

a) to reduce the number of probes used to screen for actual representation of the mRNA encoding the consensus peptide motif of interest;
b) to reduce the number of PPEs tested for immunological cross-reactivity with SPPCs on the target tumor of interest;
c) to reduce the number of immunologically cross-reactive PPEs used in in vitro, pre-clinical and clinical testing.

It is to be understood that if more than one such criterion is applied, the broader criterion is to be applied at the earlier stage of screening. In accordance with this aspect of invention the criteria to be applied include:

a) propensity to bind to an SP on the basis of thermodynamic and statistical considerations;
b) the susceptibility of a given CCP to proteolytic cleavage within a proteasome;
c) the propensity to bind to an MHC of an individual or group of individuals having common HLA types.

Another aspect the invention is directed to a composition comprising a plurality of peptides, which conform to a consensus peptide motif associated with tumor associated SPPCs. The peptides can be produced by genetic engineering as described above or by peptide synthesis.

In another embodiment, the composition consists essentially of consensus conforming peptides, which are a subset of the total theoretical set of consensus conforming peptide namely those which are found within known native proteins, preferably (in the case of human tumors) human proteins.

In another aspect the invention is directed to an antigen-binding fragment, which recognizes one or more consensus conforming peptides as presented by SPs or other PPEs which present the peptide in the same fashion as SP (i.e. so as to be immunologically cross-reactive with known tumor-associated SPPCs).

In another aspect, the invention is directed to a composition of matter comprising PPEs, wherein said composition is enriched with at least one PPE having a predetermined peptide portion which shares antigenic determinants with one or more tumor cell-surface associated SPPCs of a tumor target in a mammal, such that said peptide portion renders said PPE potentially immunogenic with respect to said tumor target in such mammal.

In one embodiment it is contemplated that the PPE has a predetermined peptide portion and an associated portion, wherein the peptide portion shares antigenic determinants with one or more tumor cell-surface associated SPPCs of a tumor target in a mammal, such that said peptide portion, as presented by said associated portion, renders said PPE immunogenic with respect to said tumor target in such mammal.

In another embodiment, the composition substantially comprises at least one PPE and in another embodiment the composition comprises a plurality of different PPEs, said PPEs characterized by different predetermined peptides which singly and/or collectively render said PPEs immunogenic with respect to said tumor target.

In another embodiment of the invention, the peptide portion of the PPE shares a substantial number of cancer-specific antigenic determinants with the peptide portion of the tumor cell surface associated SPPC.

The peptide portion of said PPE is preferably predetermined in the sense that it conforms to a consensus peptide motif which is common to a plurality of different tumor-associated SPPCs. The remaining or associated portion of the PPE assists in presenting the peptide portion of the PPE in a manner in which it shares antigenic determinants with the peptide portion of the SPPC (e.g. by presenting it in the same configuration as it would be presented by the SP) such that the peptide portion of the PPE renders the PPE immunogenic with respect to the desired tumor target. In one embodiment, the PPE is itself a SPPC. In a preferred embodiment, the peptide portion of the PPE is substantially identical to the peptide portion of the tumor cell surface associated SPPC.

In another aspect of the invention, the invention is directed to methods of generating PPEs having the desired consensus conforming peptides (hereinafter "CCP"), and antigen-binding fragments specific thereto.

In yet another aspect of the invention, the invention is directed to particular CCPs.

CA 02290722 1999-12-08

In yet another aspect of the invention, the invention is directed to a pharmaceutical composition comprising PPEs wherein said composition is enriched for, or preferably predominantly comprises, PPEs, wherein the peptide portion renders said PPE immunogenic with respect to a tumor cell surface associated SPPC.

In yet another aspect of the invention, the invention is directed to method of treating a cancer subject comprising administering to the subject an amount of a composition of matter comprising a PPE according to the invention effective to palliate the cancer.

In accordance with another aspect of the invention, we identify one or more subsets of consensus conforming peptides that have, a priori, a better percentage representation of actual tumor-related consensus conforming peptides, relative to the full set of theoretical consensus conforming peptides, as well as methods of identifying these subsets. In other words, the percentage amount of CCPs that render the PPEs immunologically cross-reactive with actual tumor-related SPPCs is higher with a smaller well-chosen subset.

For example, the subset of consensus conforming peptides that actually exist within nature is one such subset and the method of identifying them is to run a search for the consensus motif on the available databases of known proteins. Suitable databases include Genbank NCBI (e.g. using the motif scan function - Stanford University, Stanford Medical Informatics Program). Other databases including well known commercially available databases provided by DIALOG, STN, etc. include the Chemical Abstracts Registry file. Human proteins are a preferred subset for human tumors proteins.

One can adopt a conservative approach by reducing the number of hits on human proteins, for example, by rationalizing the hits in terms of their potential existence within human tumor cells and plausible association with the proteasomes of such cells, etc. For example, if a given CCP was found only in proteins expressed in certain types of human cells of limited interest and not related to tumors or tumor genesis, then that CCP could be ignored, since that CCP could not be a product of proteolysis within the tumor cell(s) of interest.

Other subsets:

Peptides which have a preference for binding to one or more types of tumor-surface expressed SPs are suitable for use herein. Preferably the CCPs show a preference for binding to SPs of HSP70 family, including preferably inducible HSP72 and the HSP90 family, including preferably HSP85. In another embodiment, a smaller subset comprising the peptides that are in

common between those which bind to more than one type of SP within a family or bind to SPs within more than one family. The peptides, which are preferred for binding to SPs can be determined directly from the literature and in the manner described the literature. Reference is made to WO 99/22761 and the scientific publications on the subject therein referenced as well as, other pertinent references including Flynn et al., Nature, 353:726 (1991); Fourie et al., J. Biol. Chem., 269:30470 (1994); and Blond-Elguindi et al., (1993) Cell 75:717-728, Gething, M.J. et al. Binding sites for Hsp70 Molecular Chaperones in Natural Proteins Cold Spring Harbor Symposia in Quantitative Biology Vol. LX p 417; Gething L.M. Current Biology (1994) 4:173; Gragerov A. et al. J. Mol. Biol. (1994) 241:133; and de Crovy-Chanel A. Gene (1999) 230:163 as well as references citing these references as disclosed in various Science Citation Indices and patent citation indices references cited within those references. All references referred to herein are hereby incorporated herein by reference.

In yet another embodiment, naturally occurring CCPs or subsets thereof can be further limited to those which overlap with peptides that have a preference for binding to MHC types within a given individual or group of individuals related by HLA type.

Peptides within the preferred subset of CCPs can be randomly elongated optionally by one or two residues or more residues to generate additional variability within the population of potential CCPs to be assessed as ligands for mapping to tumor cells. For example, 7 mers that are preferred for binding to HSP72 can be generated as 8 mers or 9 mers with the additional amino acid(s) (optionally at the carboxy terminus) representing a 20-fold to 400-fold increase in the number of permutations sought to be generated. The choice, for example, of 8 mers can be limited to those found in nature or in human protein, or according to any other strategy as discussed herein.

According to another embodiment of the invention, candidate CCPs are determined by generating a set of nucleic acid probes corresponding to the starting subset of CCPs of interest, and screening of tumor cell mRNA is carried out to determine which of the probes hybridize to the mRNA, using the mRNA from one cell type, preferably at different stages of differentiation, or the combined mRNA of a plurality different tumor types. Probes which hybridize to the selected population of mRNAs can then be analyzed to determine which corresponding CCPs actually exist within the tumor in the form of proteins and therefore which should be used as the starting population of CCPs. Techniques for generating nucleic acid probes, generating

microarrays of such probes, and analyzing the products of hybridization are well known in the art. Some of these are referred to below.

In one aspect of the invention, probes, for example, consisting of 27 nucleotides (representing 9 mers), which hybridize to larger segments of mRNA from a cancer cell (or cDNA of the mRNA) to form duplexes can be cleaved or degraded so as to remove the single stranded parts of mRNA (or cDNA) and the remaining duplex material can be used (optionally with some amplification) to generate double stranded DNA for insertion into expression vectors to generate a population of PPEs or peptides, which include the set of specific peptides within the cell that correspond with a consensus peptide motif.

In a preferred embodiment, the mRNAs are first fractionated on the basis of charge and/or molecular weight. In the case of different molecular weight fractions, it is possible, based on the available databases of proteins and their molecular weight to quantify a priori, the number and size of such fractions which are most suitable to accommodate the anticipated frequency of binding of probes and thus enhance specific binding.

In a preferred embodiment, for example, where the consensus peptide motif corresponds to the preferred binding motif of an SP found on the surface of tumors cells (for example HSP72 and HSP85 both recognized by H11), the preferred motif can be used so as to have the greatest representation within peptides degraded in proteasomes, for example HyX HyX HyX Hy, (where Hy is a hydrophobic amino acid and X is any amino acid) to which can be added at least X or XX at one or both ends. This motif is found in many human proteins.

Without being bound by any one theory, this motif is believed to be widely represented on the surface of a variety of different tumor cells for one or more of the following reasons:

a) its prevalence within human proteins (based on the formula provided below it is estimated, a priori, that this motif would occur approximately once within every protein of approximately 500 amino acids in length;
b) its propensity for binding to SP;
c) its propensity in some forms (e.g. perhaps a single aromatic series of aromatic hydrophobic amino acids) to resist proteolytic cleavage;
d) the possiblity that several degraded proteins can be represented within a proteasome so that there is a greater likelihood for the hydrophobic motif to be represented within the population or peptides that surface on SP.

The HyX HyX HyX H motif broadly represents many different hydrophobic motifs in that X can be Hy. Because it can be unnecessary for each Hy to be present for binding to SP (depending on the strength of other intermolecular interactions) this motif accounts for many hydrophobic motifs within a cell. It is noteworthy that if all X are Hy, then Hy is accounted for and can be any other amino acid.

In another embodiment of the invention, CCPs corresponding to an Zmer of repeating HyX units wherein Z is preferably 7 to 21, more preferably 7 to 15, can be used in which a series of 7 mer permutations are systematically represented to generate a repeating motif such as HyX HyXHyXHy. Thus, for example, a 9 mer will represent at least 2 series of 7 mers in the $NH_2 \rightarrow COOH$ orientation and two series 7 mers in the $COOH \rightarrow NH_2$ orientation. Similarly an 11 mer will represent 3 series of 7 mer HyX repeating units in the $NH_2 \rightarrow COOH$ direction and 3 in the opposite direction. Accordingly, this reduces the number consensus conforming peptides required to create a series of relevant PPEs and several copies of the longer peptide having a series of several 7mers represented, preferably each of comparable affinity to HSP (within a given peptide) can substitute for making each of the 7mers. The relevant set of Zmers required to create the requisite subset of CCPs can be calculated according to well-known mathematical techniques.

According to the invention we provide a set of degenerate nucleic acid probes that are complementary to mRNA encoding permutations of 3 hydrophobic amino acid within 7 amino acids, wherein at least 2 and preferably three of such hydrophobic amino acids are non-contiguous. In a preferred embodiment, probes are designed in accordance with the motif X_m $(HyX)_n$ X'_m wherein m is preferably 0, 1, or 2 and n is preferably 3 or 4 and wherein, in the creation of the probes, some degeneracy (less that 100% Hy) is permitted for Hy_1 Hy_2 Hy_3 and Hy_4 such that the representation of Hy at such positions can be less than 100%. The permitted degeneracy is a function of whatever biasing occurs at X toward amino acids preferred for intermolecular interaction with SP. It is to be understood that X (preferably X_{n1}, X_{n2}, X_{n3} or X_{n4} as opposed to X_m or X'_m) can also be biased toward certain amino acids as opposed to being random and that biasing of X_n towards Hy, depending on the degree, permits a greater degree of degeneracy from Hy, in keeping with substantial preservation of an HyXHyXHyXX motif. Where the probes are designed for microarray technology, the motif HyXHyXHyX or HyXHyXHy substantially reduces the number of permutations of probes required. The choice of

probe motif depends on size and number of microarrays sought to be employed ,and the preference for larger probes for more accurate hybridization for a given set of stringency conditions. Similar considerations with respect to biasing and degeneracy from Hy apply. Accordingly, in another aspect the invention is directed to:

1) a set of probes for determining the set of actual tumor cell consensus conforming peptides corresponding to a motif of 3, and optionally 4, hydrophobic residues within 7 consecutive amino acids wherein at least two such residues and optionally three such residues are non-contiguous (subject to optional degeneracy from Hy); and,
2) a corresponding set of peptides.

In further embodiments related to biasing of Hy and Xn, where less than a full set of permutations of the motif is opted for in the design of the probes (or the selection of peptides as discussed below) it is contemplated Hy and X can be biased towards amino acids that inhibit peptide folding and preclude degradation by proteasomes. It is believed that such peptides can be preferred for survival in proteasomes and interaction with SP and will have a greater representation among surface expressed SPPCs. In particular it is contemplated that preferred residues for binding SP are those which have been demonstrated to be preferred in binding experiments, some of which can be classed in terms of steric inhibition of peptide folding (e.g. a series of non-contiguous aromatic residues) or inhibition of such folding by possible charge repulsion or a combination of steric and charge interactions at several discrete intervals so as to counteract the tendency for a hydrophobic peptide to fold. In this regard, amino acid compositions that have no predilection towards folding (weak hydrophobic interactions, no charge attractions, etc.) are also preferred. Some of these properties can be demonstrated in the peptides, which have been generated within peptide libraries for preferred SP binding and in WO 99/22761. Peptides that are less likely to be degraded in proteasomes can be ascertained, for example, by transforming a target cancer cell with a polypeptide ((HyX) nXX)m where n=4 and m is preferably 5 to 80 to determine the pattern of degradation within the cell. Different types of hydrophobic residues could be sampled within a plurality of such polypeptides to determine which such residues at which one or more of several positions within the motif cause preservation of the motif. The same could be done for the X positions. For example, ArxxxxxAr ArXArXXXAr and ArXArXArXAr could be sampled with different aromatic hydrophobic

CA 02290722 1999-12-08

amino acids and different X, initially maintaining as many parameters as possible constant for ease of interpretation of results.

As discussed in more detail below, peptides of a length and amino acid composition which are preferred for binding MHC (of the class and HLA type required depending on the mode of administration of the peptide as an immunogen and the individuals administered to) can also be used in reducing the number of PPEs required for screening. This selection can occur at the level of creation of the probes, or preferably at level of selection of among peptides candidates that have been established to have an immunological cross-reactivity with the surface of the tumor. It is contemplated that the tumors of a series of individuals having common HLA types can evaluated contemporaneously.

According to various aspects of the invention involving pre-selection among the entire set of theoretical CCPs, it is contemplated that a predetermined peptide according to the invention is a product of selection at one or more and preferably all of the following levels of screening:

- identification (via database) of the universe of CCPs that exist within a human cell;
- design of nucleic acid probes corresponding to a set of consensus conforming peptides that are actually representative of the proteins being expressed in a tumor cell;
- identification of peptides, among the pool of consensus conforming peptides generated through nucleic acid hybridization screening, which are immunologically cross reactive with the surface of a tumor;
- identification of peptides which are determined to have predisposition for binding to the surface of the tumor based on an analysis of actual SP peptide complexes from various tumors which are identified by H11 in accordance with the purification procedures outlined below or alternative procedures for identifying the peptide portion of a substantially purified SPPC;
- HLA typing; or
- identification among the preceding groups of peptides, in the form of PPEs which cause an immune response to a target tumor, e.g. in a cytotoxic T-cell assay.

It is also contemplated that screening can be initially carried out with any of the reduced or more preferred group of CCPs contemplated herein and expanding thereafter as necessary.

It is also contemplated that the starting subset of CCPs of interest can be systematically broken into more manageable populations by fixing one or more parameter systematically. For

CA 02290722 1999-12-08

example, in the case consensus peptide motif involving a series of hydrophobic amino acids, it can be ascertained (see Fourie et al., 1994) that aromatic hydrophobic amino acids are more important for binding. It can also be ascertained from the literature (see WO 99/22761, Sloan Kettering) that other amino acids are preferred for binding to SPs.

Accordingly, as discussed above, various criteria can be employed for controlling the variation to obtain a predefined number of starting of CCPs, the number being dictated by the currently available high throughput screening technology and the extent of the effort applied to each stage of the systematic screening process. For example in the case of the hydrophobic motif HyX HyX HyX Hy such criteria can include excluding certain amino acids from being represented as X, for example negatively charged amino acid and/or amino acids that impart structure such as cysteine, glycine and proline. Another criteria for selection of X can be amino acids that are preferred for intermolecular interactions such as tyrosine, histidine, glutamine, asparagine, lysine, etc.

It is also contemplated that standard mathematical techniques can be employed to identify other consensus sequences that have a strong representation within tumor cells in terms of the frequency of the occurrence within native human protein. It can be first ascertained mathematically how long a protein would be required to have the selected motif occur randomly within the protein and the motif can be adjusted to correspond to occurrence within 25% to 100% of all such catalogued proteins, bearing in mind that several proteins might be represented within the proteosome as discussed above. This can be confirmed within a database of human proteins. Accordingly, the invention contemplates that a motif representing about a 25% to 100% occurrence within all catalogued proteins can be selected having regard to number and size of array that can be reasonably screened with the currently available technology. The number of permutations of the motif that can theoretically exist and the well known average protein size allows for the design of a motif that is anticipated to occur within a length of polypeptide which is 1 to 4 times greater than the average protein size. This can be confirmed in a database of catalogued human proteins. The levels of screening and inclusion and exclusion criteria referred to above can also be applied to such motif. The term motif contemplates known categories and sub-categories of amino acids, such as hydrophobic including hydrophobic aromatic (F, W, Y) other hydrophobic (A, I, L, M, V) or neutral-weakly Hydrophobic (A, G, P, S, T), uncharged (S, T, N, Q), positively charged (K, R, H), negatively charged (E, D) (see Lodish et al and the

brochure Protein Sequences on STN Available from CAS), as well as artificially created such categories. Artificially grouped amino acids are grouped with a view to designing a consensus peptide motif that is likely to be represented on the surface a cancer cell, as discussed below. The percentage representation of each of the categories or sub-categories of amino acids within the total of 20 can be used to calculate the number of permutations required to establish the broadest unreduced set of theoretical CCPs.

It is to be understood that there will be a large overlap between the sets of consensus conforming peptides ("CCP") identified, for example, using a DNA microchip, from individual to individual. This is expected because a given type of cancer cell is expected to produce at least for the most part, the same proteins. Accordingly, even though it might be expected, based on the literature (Srivastava et al.), that the individual CCPs on the surfaces of cancer cells from individual A will be at least somewhat different than those of individual B, the permutations of CCPs that need to be represented in the set of CCPs that accommodate individual A, will not be substantially different from the permutations that would be required to accommodate individual B and any individual differences will likely represent a small percentage of the total permutations. Accordingly, the permutations corresponding to these individual differences can readily be added to the total set of CCPs to be tested. These individual differences and differences in frequencies of occurence of common peptides might also provide some indication of differences between the tumor related biochemistry of individual A and individual B. Further such information could be expanded obtained by determining the nature of the peptides that are cross-reactive with the surface of the cell and the actual cellular proteins from which these are derived. Accordingly, such comparisons between different individuals with the same cancer type afford a method of analyzing differential tumor related protein expression and tumorogenesis.

As previously stated, it is contemplated that the set of relatively unidentifiable ligands are mapped to <u>at least one</u> set of identifiable ligands. As suggested above, the set of identifiable ligands may be designed on the basis of the following criteria:

1. the probability of occurrence of a permutation of the consensus peptide motif within every given human protein. It is desirable that the probability of occurrence of at least one such

permutation exceed 10%, more preferably 20%, more preferably 30%, more preferably 40%, more preferably 50%, more preferably 60%, more preferably 70%, more preferably 80%, more preferably 90%, more preferably 95%, more preferably 99% or greater. From another perspective, the choice of motif can be made on the basis of the predicted number of occurrences of the motif within the target size protein of interest, for example, the average size protein. The predicted number of such occurrences may be less than once in the average size protein (in the sense that the motif will only be predicted to occur within every second, third, fourth, fifth, sixth etc such size protein which would be sufficient, for example, if the corresponding number of proteins were represented within a proteasome) or greater than once, optionally from about 1 to 15 times, preferably 1 to 7 times, more preferably, 1 to 3 times. It is to be understood that a motif design opting for a greater such probability or number of occurrences is to be counterbalanced by the consequence of having to create a greater number of permutations of identifiable ligands for evaluation. It is also contemplated that where the motif of interest is one that is selected to occur in a plurality of copies or permutations within an average size protein, that, at least in the case of a DNA chip, that several passes over a set (on or more) of chips representing substantially all permutation of such motif may be required to identify all such permutations. Alternatively, or additionally, it also contemplated that one than one cDNA copy of the mRNA is made available to permit hybridization of the various copies of the cDNA at different locations on the chip, especially if mRNA of a plurality of different cells is used for creating the cDNA for the hybridization. It is also contemplated that the cDNA can be digested prior to the hybridization with restriction enzymes selected on the basis that they are not anticipated to cause any significant digestion within the motif of interest, so as to facilitate hybridization under stringent conditions and obviate the need to have several copies of the cDNA to identify all CCPs within a single such cDNA.

2. the liklihood of preservation of the consensus peptide motif in the course of proteolytic cleavage within the proteasome;

3. the propensity to bind to stress proteins; and optionally

4. the propensity to bind to MHC.

As contemplated above, it is possible to determine which amino acid residues at which locations within the consensus peptide motif will provide greater assurance that the motif will be preserved on the surface of a cancer cell after the protein in which the motif is represented passes through the proteasome. For example, it is contemplated that some hydrophobic amino acid residues will assist in preserving the motif. It is contemplated that the motif can have at least one hydrophobic amino acid at the beginning or at the end or at both ends, and optionally also one or more hydrophobic residues therebetween; hyrophobic residues optionally including particularly amino acids which are aromatic.

If cleavage sites within the proteasome were assumed to be random, the probability that a given motif will not be preserved on the surface of the cancer cell can be calculated as a function of the number of amino acid residues within the motif. The longer the motif the more likely it would be cleaved. In this connection is contemplated that a motif design based on fewer fixed amino acids (ie. non-X, for example just 2 such amino acids eg. $HyXHyX_n$ or X_nHyXHy where Hy is an aromatic amino acid and n is 3 to 15, preferably 5 to 8, and X is any amino acid and therefore is not fixed) will result in a lower probability of cleavage. From the standpoint of reducing the likelyhood of cleavage, ideally, ignoring momentarily, the rules that might be applied to the propensity to bind to HSP, the fixed positions should be set to occur adjacent to one another eg. relating back to the previous example $HyHyX_{N+1}$. In effect the latter strategy contemplates that the number of non-fixed positions are reduced and/or made more adjacent to one another, and that the criteria as to how many amino acids are part of the motif at each amino acid position within the motif are relatively more restricted to compensate for increasing the variability to X within a greater number of positions. For example rather than choosing a motif where amino acids at positions 1, 3, 5, and 7 are each chosen, for example from groups of eight (eg. barring considerations of propensity for HSP binding, any 8 amino acids) so that (assuming positions 2, 4 and 6 can be any amino acid) the number of probable occurrences of such motif within a stretch of say 280 amino acids is $(280+7) \times (8 \div 20)^4$ = approximately one (see formulas below) each of positions 1 and 2, for example, can be chosen from only 3 or preferably 4 amino acids, while the remaining 5 positions could be any amino acid (i.e. fewer fixed positions with greater

restrictions), so that the number of probable occurrences of such motif within a stretch of say 280 amino acids is $(280 \div 7) \times (3 \div 20)^2$ in the case where position 1 and 2 is chosen from 3 amino acids and $(280 \div 7) \times (4 \div 20)^2$ in the case where position 1 and 2 is chosen from 4 amino acids, both of which will equal approximately one (for the purposes of illustration). The likelyhood that two adjacent amino acids would be cleaved in the course of cleavage in, for example, 8 mer lengths, is significantly less. It is also contemplated that a motif such as $HyXHyX_n$, where N is chosen to represent the predicted sizes of the peptides brought to the surface of the cell, where N is equal to or greater than the smallest such size, will be assessed on a database of proteins to determine which species of, for example, Hy is most frequently represented in such motif. It is also contemplated that a motif wherein X or XX, etc. occurs at both the beginning and end of the motif, e.g. XXXHyHyXXX, is preferred in one aspect of the invention, since this obviates the consideration of placing certain residues such as frequently occurring hydrophobic amino acids at the beginning or end of the motif.

The invention is also directed to a population of probes conforming to the motifs generally and specifically described in the preceding paragraph.

It is also contemplated that a motif design based on the probable occurrence of several permutations of the motif would make it less likely that all such permutations of the motif will be cleaved. For example $HyHyX_{N+1}$ occurring twice, 3 times, etc. It is also contemplated that several such motif (hence the phraseology "at least one" can be represented on different respective sets of chips and that using several such sets of chips will assure that a greater number of patients' cancer cells will be able to be accurately mapped to particular CCPs. This prospect would make it less likely for each motif to be lost due to cleavage, on the one hand, and make it possible to design motifs with smaller number of permutations, on the other hand, and to use as many chips as are incrementally necessary to increase the percentage of patients that are able to be typed to particular CCPs.

As suggested above, it is contemplated that the number of amino acids that are desired to be grouped together as potential candidates for each amino acid position within a designed consensus peptide motif, could be chosen to control the probable occurrence of the motif within

CA 02290722 1999-12-08

an average size protein. Alternatively, to assure occurrence of the motif on the surface of any given cancer cell or the vast majority of cancers tested, the choice of grouping could be made on the basis of the controlling the probable occurrence of the motif with the smallest size protein that might be found within the proteasome. This more conservative approach with respect to the choice of the target size protein might be more or less appropriate based on the rigour of the design and/or conservatism of assumptions, in other respects, including 1) the number of different motifs anticipated to be tested; 2) the limitations, if any, of the current high throughput screening technology as regards the number of permutations of the motif that be practically tested for tumor matches (in accordance with the scale of the effort sought to be applied in the initial & subsequent stages of screening); and 3) the assumption made about the number of different proteins that might be represented within a proteasome; and 4) the rigor or conservatism applied in choosing the number of probable occurrences of permutations of the motif in the target size protein. This latter choice is assessed on the basis of the mathematical relationship:

$$P = M \times (0.05Q_1 \times 0.05Q_2 \times 0.05Q_3 \times 0.05Q_4 \times 0.05Q_5 \times 0.05Q_6 \times 0.05Q_7 \times 0.05Q_N)$$

Where P is the predicted number of occurrences of the motif within a stretch of amino acids of a length which is M X N, where M X N is number of amino acids in the target size protein and N is the number of amino acid positions in the motif and Q is the number of candidate amino acids within the group for each respective amino acid position $Q_1, Q_2, Q_3, \ldots Q_N$ within the group. The multiplier .05 is an alternative expression for dividing by 20 (see example above).

For ease of reference the expression $(0.05Q_1 \times 0.05Q_2 \times 0.05Q_3 \times 0.05Q_4 \times 0.05Q_5 \times 0.05Q_6 \times 0.05Q_7 \times 0.05Q_N)$ will be represented hereafter by the letter L.

In terms of the probability of occurrence of one or more permutations of the motif within the target size protein of interest, one formula that may be applied is:

$$Pr = 1 - (1 - L)^M$$

The probability that a given motif will be represented a specific number of times (G times) is given by:

$$Pr = (L^G \times (1-L)^{(M-G)}) \times M!) \div (G! \times (M-G)!)$$

Due to the ability of HSPs and MHCs to bind to diverse different peptides it is contemplated that a variety of different motifs could be designed to obtain a valuable starting set of peptides that are candidates for the methods of mapping defined herein. It is to be understood that the appropriateness of a designed or selected motif can be assessed using a database of human proteins, for example, to determine the number of occurrences of that motif in the total number of proteins within the database, preferably also taking into account the size of such proteins.

It is to be understood that any combination of the above strategies that are not practically or logically mutually exclusive can be employed for selecting a comprehensive or partially comprehensive (preferably in systematic part) set of CCPs. It is to be understood that the most important criteria according to the invention are:

- Potential existence of the peptide within nature;
- Potential existence of the peptide within the tumor cell determined preferably through nucleic acid hybridization according to well known techniques (see for example, Immunological Method Manual, Ivan Lefkovits and references listed including the references listed at p.423;
- Preferred binding to SP based on thermodynamic considerations (including resistance to hydrophobic folding) as evidenced by peptide library panning or other such studies according to technique and/or data published in the art;
- Conservation of certain types of hydrophobic amino acid sequences within proteasomes; or
- Those that have a preference for binding to H11. Method: Peptide library panning.

In accordance with another aspect of the invention, we provide, among others known in the art, a method of identifying one or more candidates for specific peptide antigens that conform to a consensus peptide motif associated with tumor surface expressed peptide antigens, including the steps of:

A) identifying at least one candidate tumor (preferably a population of such tumors) having on its surface at least one specific tumor-associated peptide antigen that conforms to said motif;

B) identifying a population of consensus conforming peptides which includes a suitable plurality of candidates for said specific tumor-associated peptide antigen;

C) identifying a population of antigen-binding fragments wherein there are a plurality of candidate fragments each characterized in that they bind to at least one specific consensus conforming peptide as presented by a PPE; and

D) screening using the candidates identified in steps A, B & C to identify consensus conforming peptides as presented by PPEs (optionally SPs) that bind to antigen-binding fragments that bind to the candidate tumor(s).

One embodiment of the invention provides a subset of a combinatorial library of antigen-binding fragments which bind to SPPCs (but not the SP itself) on one or more tumor tissues or microarray of such tissue. For example, a naïve library of antigen-binding fragments can be used to obtain anti-SPPCs by panning against a target tumor and assessing competitive binding with H11 (or an H11 related antibody), whereupon those fragments can be individually or collectively used to determine which among a population of PPEs representing a desired set of CCPs bind to those antibodies. Therefore, we provide a method to identify a substantially reduced subset of CCPs corresponding to each antigen-binding fragment, which binds to a tumor surface expressed HSSPC found on a given tumor.

According to another embodiment of the invention it is possible to immobilize the aforesaid subset of binding fragment (see for example WO 99/19506, WO 99/06834, WO 99/278745, WO 99/31509) and screen with a plurality of PPEs representing a large set of CCPs.

It is possible to map a population of immobilized CCPs to a population of tumor surface SPPCs by identifying one or more, and preferably a microarray of such tumors which express SPPCs using a population of antibodies which compete with H11 for binding to tumor expressed SPPCs. Preferably the tumor tissues and candidate PPEs are both immobilized and a large naïve library is used to map each tumor to one or more PPEs. Methods of screening proteins immobilized microarrays are referred to for instance in Lueking et al. (1999) Anal. Biochem. 270:103-111; Bradbury (1999) Tibtech 17:137-138; Moch et al. (1999) Am. J. Path. 154:981-986; Watkins et al. (1998) Anal. Biochem. 256:169-177; and van Dijk-Wolthuis (1999)

Bioconjugate Chem. HSPs can be immobilized in numerous copies and loaded with a set of CCFs selected according to the invention. Techniques for immobilization are well known in the art. Some of these are described in sources immediately above. Reference is also made to Takenaka et al. J. Biol. Chem. 270:19839-19844 (1995) which describes a method of determining when peptides are loaded onto HSPs.

Accordingly, in a general aspect of the invention, we provide a method of mapping a set of relatively unidentifiable ligands to a diverse set of identifiable ligands, said set of relatively unidentifiable ligands comprising at least one relatively unidentifiable ligand (preferably a plurality of such relatively unidentifiable ligands), said method comprising the steps of using a combinatorial library of binding molecules to bind to one of said sets of ligands to obtain a subset of binding molecules corresponding to at least one of said relatively identifiable or unidentifiable ligands and providing at least one binding molecule from said subset of binding molecules for use in binding to said other set of ligands in order to identify at least one identifiable ligand corresponding to an unidentifiable ligand.

The term identifiable in contradistinction to relatively unidentifiable without limiting the generality of these terms; can be better understood, by way of example, to mean more readily diversified, isolated or characterized, when compared with the relatively unidentifiable ligand. Without limitation, it is contemplated that isolation, diversification and/or characterization may be relatively facilitated in virtue of mapping the relatively unidentifiable ligand to one or more identifiable ligands, in the sense that:

a) the relatively unidentifiable ligands are of one type and the population of identifiable ligands is simply of another pre-determined type which is readily diversified in the sense that the minimum requisite breadth of variations can be created (e.g. facilitates larger scale mimitope creation). However, it is contemplated that the invention extends beyond high throughput advantages or use of a preferred diversity set of molecules or a preferred ligand type, for example, where one or more factor contributing to identification are more optimal, as explained in points b) through f);
b) the identifiable ligands form a relatively precise parameter set of molecules, i.e. in virtue of the identifiable set being predefined in variability according to one or more such parameters, they are, for example, more readily amenable to systematic or rapid analysis, e.g. where the method of analysis is geared to those parameters;

c) the identifiable ligands, in contrast to the relatively unidentifiable ligands, are adapted to be identified, for example, in virtue having a component moiety which contributes to a detectable phenomena when bound by the binding molecule, or in virtue of the variable portion being readily separated or isolated;

d) the identifiable ligands are relatively free of associated complexity, for example, a free standing molecule as opposed to a molecule that is complexed or otherwise associated with other molecules or structures, e.g. cell surface antigens;

e) the identifiable ligand is relatively more isolated in a particular location, e.g. for ease of access and/or analysis, for example, in a particular well, or a particular location, on a substrate;

f) the identifiable ligand is not only isolated in a particular location but the ligand at that location is known so that identification readily follows from binding of the binding molecule at that location.

For example, the variable portion of the ligand may be easily separated (e.g. the peptide portion of an SPPC) for analysis.

In one embodiment of the invention, the relatively unidentifiable ligands and identifiable ligands are related for example, in the sense that they are the same type of molecule, e.g., PPEs and tumor surface SPPCs.

In another embodiment of the invention, the binding molecules are on the whole, pre-disposed to bind to the identifiable or relatively unidentifiable ligands or both.
It is contemplated that a given binding molecule may recognize a population of closely related identifiable ligands corresponding to any given relatively unidentifiable ligand under certain conditions of binding stringency, and may recognize fewer or more such ligands under different such conditions. Accordingly, several round of panning with the population of binding molecules under different conditions of stringency may be desirable depending on the goal of obtaining more or less candidate identifiable ligands, having regard to the additional levels of screening contemplated, if any. Furthermore, several rounds of panning using one of both sets of ligands may be desired to tackle a larger set relatively unidentifiable ligands (well in excess of two), in order to obtain a many to many correspondence in a first pass or set of passes, and then to focus on individual one unidentifiable ligand (mapped to a plurality of identifiable ligands or a minimum number of identifiable ligands), in succeeding passes. Furthermore, it may be

desirable to use subsets of binding molecules in individual passes as part of a systematic or partially systematic effort, for example, in order to use a more pre-determined set of binding molecules (in terms of a given parameter), or in order to use a more conservative approach at the outset, or simply to use different libraries in different passes.

It is also contemplated that one or both sets of the ligands may be immobilized, e.g., in a microarray, for promoting greater binding specificity or automation.

It is also possible to immobilize a full naive set or particular subsets of binding molecules, for example, subsets that have been determined to be tumor specific or ligand-type specific, and to carry out the mapping in one location, for example, in multiple passes, each time using a full set of both populations of ligands sought to be compared or relatively fewer passes (possible one) with multiple copies of the different ligands within the respective sets represented in a given round of panning.

In a preferred embodiment of the invention, the relatively unidentifiable ligand is relatively difficult to isolate and characterize in virtue of its association with a cell surface, for example, tumor surface antigens, and the relatively identifiable ligands are a set of potential peptide antigens. In preferred embodiments, the identifiable ligands are relatively identifiable in the virtue of the greatest number of factors, which contribute to the ease of identification e.g. precise diversification according to certain parameters, as well as relative isolation and ease of characterization. Optimally, in the case of HSPs presenting consensus conforming peptides, each desired permutation is known and assigned to a particular location on a substrate. Minimally, identifying individual relatively identifiable ligands may be enhanced, for example, in virtue of a threshold amount of diversity and some enhancement in isolation or independence from associated complexity.

The terms binding molecule and ligand are intended to be limited only in terms of the pragmatic import of the mapping method, namely so that the binding molecule is used to identify in a selective manner, identifiable ligands from a diverse set corresponding to at least one, but advantageously many, relatively unidentifiable ligands, so as to identify candidate identifiable ligands (often the minimal number in excess of one depending on the additional screening contemplated) corresponding to a given individual relatively unidentifiable ligand. With this caveat in mind, certain scope can attempted to be given to these terms.

The term "binding molecule" can refer to a molecule of sufficient size and complexity so as to be capable of selectively binding a ligand. Such molecules are generally macromolecules, such as polypeptides, nucleic acids, carbohydrate or lipid. However, derivatives, analogues and mimetic compounds as well as small organic compounds are also intended to be included within the definition of this term. The size of a binding molecule is not important so long as the molecule exhibits or can be made to exhibit selective binding activity to a ligand. For example, a binding molecule can be as little as about one or two, and as many as tens or hundreds of monomer building blocks which constitute a macromolecule-binding molecule. Similarly, an organic compound can be a simple or complex structure so long as selective binding affinity can be exhibited.

Binding molecules can include, for example, antibodies and other receptor or ligand binding polypeptides of the immune system. Such other molecules of the immune system include for example, T cell receptors (TCR), major histocompatibility complex (MHC), CD4 receptor, and CD8 receptor. Additionally, cell surface receptors such as integrins, growth factor receptors and cytokine receptors, as well as cytoplasmic receptors such as steroid hormone receptors are substantially also included within the definition of the term binding molecule. Furthermore, DNA binding polypeptides such as transcription factors and DNA replication factors are likewise included within the definition of the term binding molecule. Finally, polypeptides, nucleic acids and chemical compounds such as those selected from random and combinational libraries are also included within the definition of the term so long as such a molecule exhibits or can be made to exhibit selective binding activity toward a ligand.

As used herein, the term "polypeptide" when used in reference to a binding molecule or a ligand is intended to refer to peptide, polypeptide or protein of two or more amino acids. The term is similarly intended to refer to derivatives, analogues and functional mimetics thereof.

The term "ligand" can be understood to refer to a molecule that can be selectively bound by a binding molecule. A ligand can be essentially any type of molecule such as polypeptide, nucleic acid, carbohydrate, lipid, or any organic derived compound. Those skilled in the art know what is meant by the meaning of the term ligand. Specific examples of ligands are the tumor antigens described herein which are selectively bound by the human antibody binding molecules described in the examples.

As used herein, the term "diverse population" is intended to minimally refer to a group of two or more different molecules.

As used herein, the term "selective" or "selectively" when referring to the binding of a binding molecule to a ligand is intended to mean that the interaction can be discriminated from unwanted or non-specific interactions. Discrimination can be based on, for example, affinity or avidity and therefore can be derived from multiple low affinity interactions or a small number of high affinity interactions. For example, a binding molecule interaction with a ligand is generally greater than about 10^{-4} M, is preferably greater than about 10^{-5} M and more preferably greater than about 10^{-6} M. High affinity interactions are generally greater than about 10^{-8} M to 10^{-9} M or greater. Unless otherwise stated, selective binding is implied. It will be appreciated that selective binding is necessary to identify immunologically cross-reactive SPPCs or MHC peptide complexes (i.e. those that share antigenic determinants with tumor surface species) that will be useful to generate a T-cell response.

As used herein, the term "immobilizing" or grammatical equivalents thereof, refers to the attachment, as through the binding of a population of binding molecules, to a solid support. Immobilization can be through specific interactions with the binding molecule and an agent on the solid support. The agent can be, for example, a chemical moiety, which allows covalent or non-covalent interactions sufficient to hold the population of binding molecules to the solid support. Immobilization can also be through tethers or linkers. Such linkers can be covalent linkers, hydrolyzable linkers, photo-labile linkers or other linkers that allow the binding molecules to be selectively attached. Linkers can also be polypeptides or other biomolecular linkers such as antibodies, lipid attachments, streptavidin, receptors, fusion polypeptides, or any biomolecule that can tether the binding molecule to the solid support. Additionally, domains of polypeptides can similarly be linkers. For example, hydrophobic domains which allow direct absorption to a plastic due to specific sequences, which are molecular tags or recognition sequences can be linkers for binding polypeptides.

As used herein, the term "solid support" refers to a solid medium, which is sufficiently stable so as to allow immobilization of a population of binding molecules. Solid supports can include, for example, membranes such as nitrocellulose, nylon, polyvinylidene difluoride, plastic, glass, polyacrylamide or agarose. Solid supports can also be made in essentially any size or shape so long as it supports the immobilization of a population of binding molecules. For

example, the solid support can be a flat planar surface such as a natural or synthetic membrane filter or a glass slide. Alternatively, the solid support can be of various spherical shapes, including, for example, beads made of glass, polyacrylamide or agarose. Porous mediums can similarly be used as solid supports and such mediums are included within the definition of the term as used herein. Additionally, any of the solid supports can be modified, for example, to include functional chemical groups that can be used directly or indirectly for attachment of binding molecules or linkers. It is contemplated that multiple HSPs or MHCs can be immobilized on a solid support and later loaded with peptides either at random or pre-defined locations.

The term "antibody" can be understood to mean a polypeptide, which binds to a ligand and is intended to be used consistently with its meaning within the art. The term immunoglobulin is similarly intended to fall within the scope of the meaning of the term antibody as it is known and used within the art. The polypeptide can be the entire antibody or it can be any functional fragment thereof which binds to the ligand. The meaning of the term is intended to include minor variations and modifications of the antibody so long as its function remains uncompromised. Functional fragments such as Fab, $F(ab)_2$, Fv, single chain Fv (scFv) and the like are similarly included within the definition of the term antibody. Such functional fragments are well known to those skilled in the art. Accordingly, the use of these terms in describing functional fragments of an antibody are intended to correspond to the definitions well known to those skilled in the art. Such terms are described in, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989); Molec. Biology and Biotechnology: A Comprehensive Desk Reference (Myers, R. A. (ed.), New York: VCH Publisher, Inc.); Huston et al., Cell Biophysics, 22:189-224 (1993); Pluckthun and Skerra, Meth. Enzymol., 178:497-515 (1989) and in Day, E. D, Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc., New York, NY (1990), which are incorporated herein by reference.

In one embodiment of the invention, said set of identifiable ligands and said set of relatively unidentifiable ligands are antigenically related, for example, PPE and tumor surface SPPCs. In this case, optionally said library of binding molecules is specific for said set of identifiable ligands as discussed below.

In a preferred embodiment of the invention, the binding molecules serve to identify identifiable ligands, which are immunogenically cross-reactive with said relatively unidentifiable ligands.

Accordingly, in a more specific general aspect of the invention, we provide a method of mapping a set of identifiable ligands (e.g. a population of PPEs) to a set of relatively unidentifiable ligands (e.g. tumor antigens), said set of relatively unidentifiable ligands related to said first set, said set of relatively unidentifiable ligands comprising at least one relatively unidentifiable ligand, said method comprising the steps of using a combinatorial library of binding molecules to bind to one of said sets of ligands to obtain a subset of binding molecules corresponding to at least one of said relatively identifiable or unidentifiable ligands and providing at least one binding molecule from said subset of binding molecules for use in binding to said other set of ligands in order to identify at least one identifiable ligand corresponding to an unidentifiable ligand. Preferably said set of identifiable ligands and said set of relatively unidentifiable ligands are antigenically related. More preferably said library of binding molecules is specific for said set of identifiable ligands as discussed below.

In another embodiment of the invention, the library of antigen binding fragments used for mapping the relatively unidentifiable ligands to the identifiable ligands is a single variable domain library, preferably a heavy chain variable domain library. Reference is made to U.S. patent nos. 5,702,892; 5,759,808; 5,800,988; 5,840,526; 5,874,541, and to Lauwerey M. et al., EMBO Journal, 17(19), p.3512 (1998); Reiter Y, J. Mol. Biol. (1999) 290:3 685-698. In the case of well-known camelid type single domain antibodies disclosed in several of the previously mentioned patents, the candidate binding fragments have loop structures which are useful for binding into cavities (see also Muyldermans S. et al. J Mol. Recognit. (1999) 12(2) 131).

Reference is also made to our co-pending U.S. patent application filed November 4, 1999, entitled "Enhanced Phage Display Libraries and Methods for Producing the Same", the disclosure of which is hereby incorporated by reference. This application discloses a variable heavy chain domain library based on the heavy chain variable region of the antibody designated A6 which spans from a position upstream of FR1 to a position downstream of F4. A6 has a CDR3 of 23 amino acids in length, which forms such a loop structure. Furthermore, in one embodiment of the A6 library, positions 44, 45, and 47 (Kabat numbering) may be substituted by non-hydrophobic amino acids, for example, those residues described in the literature on camelid

single domain Abs (see Lauwercy et al. 1998, and U.S. Patent Nos. 5,840,526 and 5,874,541). Furthermore, in the absence of those substituted residues, this library surprisingly produces truncated loop structures, which can be advantageously be used for binding to the antigen binding sites of antibodies and may be adapted as described herein to SPPC binding sites. Other such binding fragments include libraries of cysteine noose peptides (see WO 99/23222).

Such loop structure based libraries can be varied to present random or partially random loop permutations (excluding for example residues that impart configuration [e.g. glycine or proline] or biasing in favor of amino acids such as hydrophobic amino acids in some positions or those that are preferred for intermolecular interaction) that are varied not only in amino acid composition but also in the size of the loop. Alternatively variations in the size of the loop can be generated in a second round of panning with candidates that have preferred amino acid compositions determined from a first round of panning. These libraries and method can be used to create peptide-specific SPPC binding molecules in the sense that they collectively recognize a substantial diverse population of different SPPCS and individually preferably a minimum number of such SPPCs, preferably less than 15, more preferably less than 10, more preferably less than more preferably less than 3, more preferably 1. Reference is also made to the methods of WO 99/120749, which can be adapted to create peptide specific anti-SPPC binding fragments using H11. It is also contemplated that anti-HSP antibodies can be generated by panning against SPPCs and subtracting from the population of binders those that bind to the SP alone. Reference is made to our co-pending PCT application entitled "Antigen Binding Fragments Specific For Tumor Associated Antigens" filed November 29, 1999, the contents of which are hereby incorporated by reference.

In another aspect of the invention, the binding region (amino acids involved in the ligand interaction) of one or more preferred peptide specific anti-SPPCs can be varied randomly or systematically in accordance with a consensus peptide motif while maintaining the flanking regions that impart configuration constant to create a library of peptide specific anti-SPPC candidates. Such libraries can be advantageously used, for example, in mapping tumor-surface SPPCs to PPEs that conform to a consensus peptide motif as defined herein.

The invention is also directed to a method of creating an HSP binding site mimitope using naïve or biased libraries of single domain variable fragments or loop structures by panning against a sub-population of SPPCs from which the peptide has been released (e.g. by ATP or

acid treatment) to obtain a sub-population of binding fragments which are HSP binding site candidates and optionally determining which among such fragments is unable to bind to the peptide loaded population of SPPCs. Such candidates may be sequenced to determine whether such fragments have sequences, which are preferred for binding to SPPCs. In a preferred embodiment of this method, the population of binding fragments are engineered to have binding portions, which contain preferred HSP binding peptide sequences as discussed above and the flanking regions of such binding portions may also be systematically varied in length to provide different loop geometries. This method can be used to generate one or more preferred HSP binding site candidates. In an ensuing step, anti-idiotypes that show preferred affinity binding to such preferred HSP binding site candidates can then be generated, for example, those that have amino acid constitutions or that are preferred for binding to the same types of peptides that are preferred for binding to HSP. These can then be tested as PPEs, for example by assessing the cross-reactivity of a particular SPPC to a corresponding PPE(s), wherein the peptide portions are identical or substantially identical. In another embodiment of the method, employing the mapping strategy disclosed herein, a population of minor variants of one or more anti-idiotypes that show such cross-reactivity can then be created using techniques such as codon based mutagenesis (see also the technique described in co-pending U.S. application filed November 3, 1999, entitled "Enhanced Phage Display Libraries and Methods for Producing the Same") such that the anti-idiotype variants are strongly biased to the parental amino acid constitutions. These closely related variants can then be used to create a population of PPEs that are cross-reactive to corresponding SPPCs using for example the anti-SPPC specific library of single domain fragments or loop structures (as discussed above) as binding fragments for mapping purposes. In this way, a variety of different specific SPPCs could be tested for cross-reactivity by loading each of the relevant peptides onto each member of a defined set of anti-idiotype variants and testing for matching pairs that bind with high affinity to a given binding fragment. For example, SPPCs pooled from tumor cell extracts of a variety of different tumors could be mapped to a set of such anti-idiotypes, preferably in multiple copies, loaded with a suitably large variety of peptides conforming to a consensus peptide motif and the candidates that map to one another can then be assessed for those that have the most closely related , and preferably identical, amino acid constitutions. Amino acid analysis of samples of such anti-idiotypes could be used to create a smaller set of useful HSP mimics that are each adapted to a specific set of peptides that are

allocated to a single array or set of arrays for screening. Moreover amino acid analysis of several such matches or prototype anti-ids could be used to create a universal HSP mimic for the consensus sequence in question, particularly for consensus sequences that are preferred for binding to an HSP, such as HyXHyXHyXHy.

Accordingly, the invention is also directed to a single variable domain anti-SPPC or a functional fragment thereof when such fragment is expressed in the form of a fusion protein with an outer surface protein residing on a phage. It is also contemplated that such fragments can be tested for their stability as loop structures (with or without a portion of the outer surface phage protein e.g. P3, but divorced from the phage particle).

The invention is also directed to a library of single variable domain anti-SPPCs including those of the type adapted to bind to individual peptide specific SPPCs.

In a more general aspect of one embodiment of the invention we provide a method of mapping a diverse set of identifiable ligands to a set of relatively unidentifiable ligands, said set of relatively unidentifiable ligands immunogenically related to said set of identifiable ligands through a (preferably SPPC related) consensus peptide motif, said set of relatively unidentifiable ligands comprising at least one and preferably a plurality of relatively unidentifiable ligands, said method comprising the steps of using a combinatorial library of binding molecules to bind to one of said sets of ligands to obtain a subset of binding molecules (at least one) corresponding to at least one of said identifiable or relatively unidentifiable ligands and providing at least one such binding molecule from said subset of binding molecules for use in binding to said the other set of ligands in order to identify at least one identifiable ligand corresponding to an unidentifiable ligand, wherein:

a. said set of binding molecules is collectively adapted to bind to substantial number of permutations of SPPCs

b. preferably, a substantial number of such binding molecules are adapted to bind to a minimum number of SPPCs in excess of one, more preferably only one. The same method could be applied to MHC peptide complexes or related sets of ligands.

The method of generating the A6 library is detailed in the co-pending patent application filed on November 4, 199 entitled "Enhanced Phage Display Libraries and Methods for Producing Same."

Without limiting the invention herein defined or being bound by any theory, it is postulated that parts of the peptides that are actually bound to the SP peptide-binding site are directly immunogenic with respect to the tumor. Thus, according to one embodiment of the invention, the entire consensus conforming peptide, or a part thereof corresponds to the consensus motif HyXHyXHyXHy.

According to another embodiment of the invention, the consensus conforming peptides correspond to peptides, which prefer to bind to H11, and consensus motifs, which are derived from these peptides.

The population of candidate consensus conforming peptides, can optionally be generated using a peptide display library. Optionally, the candidate consensus conforming peptides are presented on the surface of PPEs, preferably human tumor surface associate SPs such as SPs of 70 and 90 families. This can be accomplished according to methods known in the art (see, W) 99/22761) including such as that described in WO 99/29834. Optionally, the population of candidate consensus conforming peptides is presented on the surface of a population of professional APCs according to methods known in the art.

The population of antigen-binding fragments can be generated, by screening for candidates that bind to the population of consensus conforming peptides and creating one or more populations of variants related to one or more of such candidates according to the scheme defined below. Alternatively, the population of antigen binding-fragments can be generated by using H11 as a parental binding molecule using the scheme and methods defined below. The populations generated according to either of the preceding approaches can be pooled. H11 itself which is believed to bind to a variety of different specific consensus conforming peptides, can be used as control (e.g. via a competition binding assay) in selecting candidates for specific tumor-associated peptides antigens which conform to the consensus motif of choice, in the manner discussed herein. These candidates can be used to identify peptide antigens according to the scheme and methods defined below, as probes for various diagnostic applications, to identify new antigen-binding fragments and variants thereof for the use in the last described screening method (for isolating specific tumor-associated SPPCs by mapping CCPs to tumor using a suitable antigen-binding-fragment library), to identify different and possibly more specific consensus motifs, and to create antigenic compositions enriched for potentially relevant SPPCs, as discussed below.

The preferred library of antigen-binding fragments (as discussed below) can also be used to generate other antibodies that bind to a plurality of different SPPCs in virtue of the associated CCPs by the using the scheme described herein. These antibodies could also be used as controls by way of competition assay to ensure appropriate tumor localization to SPPCs.

In another aspect the invention is directed a phage display library which displays a plurality of antigen-binding-fragments that recognize one or more consensus conforming peptides. In one embodiment this library is created using H11 as a parental binding fragment, in the manner described below.

The invention is also directed to a population of genetic packages having a genetically determined outer surface protein, which collectively display a plurality of potential binding fragments in association with said outer surface protein, each package, including a nucleic acid construct coding for a fusion protein, which encodes at least a portion of said outer surface protein and a variant of at least one parental binding fragment, wherein said parental binding fragment binds specifically to one or more tumor-associated consensus conforming peptides as presented by a SP, wherein at least part of said construct, including preferably at least a portion of the CDR3, is only partly randomized in that it is biased in favor of encoding the amino acid constitution of said parental binding fragment such that said plurality of different potential heavy chain binding domains are on the whole adapted to be or are better capable of binding to tumor-associated consensus conforming peptides as presented by SPs. Preferably said parental binding fragment is H11. Preferably said genetic package is a phage and said soluble parental binding fragment is selected from the group consisting of an scFv, Fab, V_H, Fd, Fabc, $F(ab')_2$.

In another embodiment, the population of genetic packages or phage, comprising a plurality of libraries, which are pooled, wherein at least a first and second of said pooled libraries differ in the degree of biasing to wild-type amino acids. In one embodiment, libraries wherein the CDR3 is biased 95%, 90%, 85%, 80%, 75% and 70% in favor of the wild-type (e.g. H11) constitution are pooled.

A. SPPCs.

The invention also encompasses a substantially isolated, tumor-associated SPPC designated C-antigen. The compositions can also include physiologically acceptable excipients

CA 02290722 1999-12-08

and/or adjuvants. The invention further encompasses compositions containing substantially purified SPPC peptides and immunogenic fragments thereof.

Any of the SPPC or SPPC peptide compositions can be formulated in therapeutically or immunogenically effective amounts. These compositions can also be provided in dried or concentrated form for rehydration or dilution prior to use.

The invention is further directed to a method of isolating an intact SPPC by: fractionating a tumor cell extract to obtain a hydrophobic fraction; identifying an antigenically active fraction, thereof using an antigen-binding fragment which binds specifically to the SPPC; applying the antigenically active fraction to an ADP chromatographic media; applying the active fraction eluted from the ADP chromatographic media to a strong anionic medium; collecting active fractions eluted from the strong anionic medium where activity is determined by specific reactivity with the antigen-binding fragment; and, preferably, purifying the active fractions under non-denaturing conditions, preferably electrophoretic extraction.

The invention is also directed to a method of isolating an antigenic SPPC by: fractionating a tumor cell extract on an affinity medium, such as an immunoaffinity column, to bind the complex; eluting the complex to obtain an eluate; applying the eluate to a molecular sieve capable of separating the SP from the peptide; isolating, (and if necessary, sequencing,) the peptide; and re-associating the SP with the isolated peptide. The invention is also directed to C-antigen peptide isolated by said method and as described in more general terms below.

B Antigen-binding-Fragments.

The invention encompasses a composition of matter comprising an isolated antigen-binding-fragment specific for a SPPC. These antigen-binding fragments are termed "anti-SPPCs."

The invention further encompasses a composition of matter comprising an isolated antigen-binding fragment of an antibody specific for a tumor-associated SPPC and a physiologically acceptable excipient.

The invention also encompasses a method of obtaining antigen-binding fragments specific for a tumor-associated SPPC by generating a population of antigen-binding fragments; generating tumor-associated SPPC; screening the antigen-binding fragments with the complex to obtain antigen-binding fragments that bind specifically to tumor-associated SPPC; and screening the antigen-binding fragments obtained for cell surface tumor-associated reactivity.

As discussed in greater detail below, particularly with reference to specific Examples herein, the final screening step is preferably accomplished by screening the antigen-binding fragments obtained with at least one and preferably several cell lines derived from one or more cancer types and at least one and preferably several normal non-cancerous cell types. Suitable screening methods and parameters are known in the art and are also described in the Examples with respect to antibody H11.

C. Polynucleotides

The invention encompasses compositions containing polynucleotides encoding the antigen-binding fragments. Recombinant vectors containing the polynucleotides and host cells transfected with the vectors are also encompassed by the invention.

The invention encompasses compositions containing polynucleotides encoding the peptide portion of an SPPC. In the case of C-antigen, the invention encompasses polynucleotides encoding the peptide portion of the complex.

D. Kits.

The invention encompasses kits comprising the antigen-binding fragments of the invention and buffers, labeling agents, toxins and radioisotopes necessary for the diagnostic or therapeutic use of the antigen-binding fragments.

The invention further encompasses kits comprising the SPPC or peptide portion thereof of the invention and buffers, adjuvants etc. for the therapeutic and/or immunogenic use of the compositions.

E. Compositions.

The invention encompasses therapeutic or pharmaceutically or physiologically acceptable compositions of matter. These compositions include an active component comprised of the SPPCs, peptides, antigen-binding fragments and polynucleotides described herein and a physiologically acceptable buffer, vehicle or excipient thereof. Preferably, the active component is present in an "effective amount," that is, an amount to effect the desired result such as amelioration or palliation of symptoms or imaging. In particular, the antigen-binding fragments are suitable for inhibiting metastases. Methods of use therein and compositions for use therefor are further encompassed by the invention.

F. Methods of Treatment.

The invention encompasses methods of treating cancer patients. The methods comprise administering to the patient a therapeutically effective amount of an antigen-binding fragment of the invention. The methods further comprise administering to the patient an immunogenic amount of an SPPC or SPPC peptide of the invention.

The invention also encompasses a method of treating a cancer subject comprising administering to the subject an amount of a composition of matter comprising an isolated antigen-binding fragment specific for a tumor-associated SPPC and a physiologically acceptable excipient effective to elicit a cancer-specific immune response.

The invention further encompasses a method of treating a cancer subject comprising administering to the subject an amount of a composition of matter comprising an isolated antigen-binding fragment specific for a tumor-associated SPPC and a physiologically acceptable excipient effective to ameliorate the cancer.

G. Additional Methods of Use.

The invention encompasses methods of inducing a tumor-specific immune response in a subject. The methods can be used for cancer treatment as above, or as a preventative measure, particularly in a subject at risk for cancer. The methods include administering to the subject an amount of an active effective to induce a cancer-specific immune response in the subject. The active can be tumor-associated SPPC or an antigenic fragment thereof or an anti-idiotype anti-SPPC antibody.

The invention also encompasses methods of detecting or imaging cancer cells. In the case of in vitro detection, labeled anti-SPPCs are incubated with biological samples under conditions and for a time sufficient to allow specific binding of the anti-SPPCs to cancer cells. Unbound anti-SPPCs are then removed and bound label measured or detected. In the case of imaging, labeled anti-SPPCs are administered to a patient (either having or suspected of having cancer), or animal model system in an amount and under conditions sufficient for the anti-SPPCs specifically binding to cancer cells. Excess or non-specifically bound anti-SPPCs are removed, if necessary, and bound anti-SPPCs are detected.

The invention also encompasses compositions and methods of use thereof in diagnostic antibody clearance. Anti-SPPC can be administered to an individual who has received a labeled anti-SPPC the course of radioscintigraphy or radiotherapy to remove the label. Effective

imaging using radiolabeled antibodies is hampered due to excess circulating radiolabeled antibody, which often takes several days to clear. Accordingly, the SPPC recognized by the anti-SPPC is administered to the individual at a specified time after administration of the labeled anti-SPPC. Antigen that is complexes with the antigen-binding fragments at sites other than the tumor, such as in the circulation and interstitial spaces, promotes clearance of non-bound antibody and decreases background radiation. As a result, the level of label in unaffected tissues is reduced, and the image of the tumor (in comparison to neighboring tissues) is enhanced.

The invention further encompasses methods of monitoring progress and efficiency of anti-cancer therapy. In this case, cancer patients undergoing chemotherapy or other form of anti-cancer therapy are treated as described for diagnostic imaging but repeatedly and at defined intervals. A decrease in tumor burden as indicated by decreased antigen-binding fragment binding is indicative of successful chemotherapy.

In another aspect the invention is directed to a kit, which comprises a CCP library as presented by PPEs, and a antigen-binding fragment library for screening such CCP library.

According to another aspect of the invention, the invention is directed to antigen-binding fragments, which bind to at least one tumor-associated SPPC, and preferably to a plurality of such SPPCs that share a common consensus peptide motif. Such antigen-binding fragments are screened against a panel of different tumor types to identify positive clones which are specific for one or more tumor-associated SP peptide complexes and a correspondingly wide variety of tumor single and multi-tumor specificities. In a preferred embodiment of the invention such variants of H11 antigen-binding fragments are multi-carcinomic anti-SPPC, which bind to consensus peptide motif which is enriched for hydrophobic residues. Various embodiments of the preferred hydrophobic motifs are presented in the claims.

Alternatives to generating SP-CCP complex libraries are described herein. According to one such alternative, suitable cells are caused to uptake one or more selected CCPs transfected with nucleic acid constructs comprising a polynucleotide encoding one or selected CCPs. In the case of professional APCs, the cells are then directly screened with antigen-binding fragments as defined in the preceding aspect of the invention. Alternatively, SPPCs are then extracted from such cells, partially purified and screened with tumor-associated antigen-binding fragments according to a preceding aspect of the invention in order to identify antigen-binding fragments and corresponding SPPCs which are tumor-associated. The positive clone which shows strong

reactivity identify the selected consensus conforming peptide and link these CCPs to one or more particular tumors.

As an alternative to generating a library of SP-CCP complexes, selected consensus conforming peptides can be generated in a peptide display library or presented in the form of PPEs and screened with tumor-associated antigen-binding fragments representing a wide variety of single and multi-tumor specificities.

Preferred consensus conforming peptides are identified according to one or more of the following criteria:

(1) strongest representation in databases of human proteins;

(2) strongest representation in preferred SP binding peptides (see WO 99/22761); strongest conformity to peptides motifs which bind to H11.

VII. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts flow cytometric analysis of cells recognized by H11.

Figure 2 depicts a flow cytometric analysis of cells recognized by H11.

Figure 3 depicts binding of H11 to tumor cell extracts.

Figure 4 depicts binding of H11 to tumor cell extracts.

Figure 5 depicts binding of H11 to human tumor cell lines.

Figure 6 depicts a schematic of the expression vector pSJF1.

Figure 7 is a graph depicting mean tumor volume per day after treatment with H11 scFv (closed circles) or PBS (open circles).

VIII. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Antigen-binding fragments that bind to SPPCs are more generally described in our co-pending application No. 60/149,587.

The term "associated portion" as used herein with reference to a PPE refers to the portion of the PPE other than the predetermined peptide portion and includes a polypeptide (or other moiety) to which the predetermined peptide portion is terminally or otherwise, linked two non-contiguous portions of a polypeptide in which the predetermined peptide portion is peptide segment, an MHCI, a heat shock protein, which forms a complex with said predetermined peptide portion, etc. The associated portion can actively (by imparting conformation to) or

passively ("linkage" without imparting conformation) present the predetermined peptide portion in which it shares antigenic determinants with an SPPC, for example in the same conformation which the SP imparts to the SPPC peptide.

The term "predetermined peptide portion" refers to a peptide, for example, a 7 mer, which is selected on the basis that it has a particular amino acid sequence or constitution or a particular consensus motif. The predetermined peptide portion can be linked to any other suitable ligand provided the ligand does not interfere with antigen presentation. Ligands that enhance binding to SPs are described for instance, in WO 97/06821; and WO 99/22761.

Methods of making "DNA vaccines" using polynucleotides encoding various subjects of consensus conforming peptides according to the invention have been described for various application. Reference is made to US Patent Nos. 5,580,859 and 5,589,466, which describe the generation of nucleic acids that can by used in "DNA vaccine" applications as well as US Patent Nos. 5,843,913, 5,814,617, 5,811,406, 5,736,524, 5,676,954, 5,620,896, 5,593,972, 5,589,466 and 5,580,859. The disclosures of all of which are hereby incorporated by reference.

Methods of loading consensus conforming peptides onto antigen presenting cells, particularly dendritic cells (DCs), are described in references provided herein. Methods to isolate DCs from blood and the expansion of these *in vitro* to yield APCs for clinical use in immunotherapy is described in references provided herein.

All references disclosed in this application are hereby incorporated by reference 1) Pardoll D.M. (1998) Cancer vaccines. *Nat. Med.*, 4:525-531; 2) Young J.W. and Inaba K. (1996) Dendritic cells as adjuvants for class I major histocompatibility complex-restricted anti-tumor immunity. *J. Exp. Med.*, 183:7-11; 3) Hart I. and Colaco C. (1997). Fusion induces tumor rejection. *Nature,* 388:627-628; 4) Banchereau J. and Steinman R.M. (1998). Dendritic cells and the control of immunity. *Nature,* 392:245-252; 5) Bakkar A.B.H. *et al.* (1995). Generation of anti-melanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells *in vitro. Cancer Res.*, 55:5330-5334; 6) Pogador A. and Gilboa E. (1995). Bone marrow-generated dendritic cells pulsed with class-I restricted peptides are potent inducers of cytotoxic T-lymphocytes. *J. Exp. Med.,* 182:255-260; 7) Hsu F.J. *et al.* (1996). Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.*, 2:52-58; 8) Boczkowski D. *et al.* (1996). Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J. Exp. Med.*,

184:465-472; 9) Gong J. *et al.* (1997). Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat. Med.*, 3:558-561; 10) Murphy G. *et al.* (1996). Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate*, 29:371-380; and, 11). Nestle F.O. *et al.* (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.*, 4:328-332.

A. SPPCs

The invention encompasses compositions comprising tumor-associated SPPCs. The invention also encompasses compositions comprising substantially isolated SPPC peptide.

1. <u>Characterization</u>

It has now been found that an antigen designated "C-antigen" which is found on a variety of cancer cells but only at low levels or not at all on normal, non-cancerous cells, consists of a SPPC. This antigen was previously described by its immunologic reactivity with an antibody designated H11, but was not previously isolated or characterized. H11 is described in detail in WO97/44461. The antigen comprises the complex of SP and a peptide; H11 binding is lost when the complex is dissociated. H11 specifically recognizes a broad range of many, but not all, neoplastic cells. The specificity of H11 includes, but is not limited to, glioblastoma, neuroblastoma, malignant melanoma, breast adenocarcinoma, lung adenocarcinoma, small cell lung carcinoma, colon adenocarcinoma and prostate adenocarcinoma.

The term "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acid residues of any length. The polymer can be linear or branched, it can comprise modified amino acids or amino acid analogs, and it can be interrupted by chemical moieties other than amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; including, but not limited to, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling or bioactive component. Unless stated or implied otherwise, the term antigen-binding fragment includes any polypeptide monomer or polymer with immunologic specificity, including the intact antibody, and smaller and larger functionally equivalent polypeptides, as described herein. With respect to "stress protein-peptide complex," "peptide" refers to the peptide moiety non-covalently complexed specifically to SP.

CA 02290722 1999-12-08

Maintenance of the complex is typically ATP dependent and can be dissociated by the removal of ATP. Dissociation also occurs under denaturing conditions.

The complexed peptide is endogenous. Endogenous peptides are native peptides complexed with SPs in vivo. Native peptides can be those associated with SPs in vivo or modifications thereof including those made by associating a peptide with a SP in vitro to form a complex which is antigenically similar to that found in vivo, particularly so as to be specifically reactive with the same antigen-binding fragment. Native peptides and modified peptides can be made by recombinant DNA techniques, peptide synthesis and other methods known in the art. In vitro complex association can be obtained with peptides either isolated from a mammalian source or obtained by recombinant means. Such peptides can be isolated and sequenced by any method known in the art. These methods include, but are not limited to, those of Kassel et al. (1994) Anal. Chem. 66:236-243; and Kiselar et al. (1999) Anal. Chem. 71:1792-1801.

The invention also encompasses compositions comprising at least one SPPC, which is specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer. In particular the SPPC contains a non-covalently bound peptide, which confers the specific immunogenicity.

The invention also encompasses compositions comprising a plurality of SPPCs which are specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer. In particular the SPPCs contain different non-covalently bound peptides, which confer the specific immunogenicity.

Preferably, for the purposes of tumor-specific treatment, SPPCs are "tumor-associated." The use of disease-associated SPPCs for treatment is also encompassed by the invention. The SPPCs of interest might not be found exclusively on cancer cells but might also be found on other cells. To the extent SPPCs are on normal cells not found associated with tumors, it is at a level of detection below that of the invention. Therefore, as used herein, "not on normal cells" indicates that the SPPCs have not yet been detected on normal cells. However, normal cells could express SPPCs if diseased. In this sense, the term "tumor-associated" complexes encompasses both tumor-specific and disease-associated. Accordingly, it is contemplated that a SPPC and/or an antigen-binding fragment specifically reactive therewith, obtained according to the methods defined herein, can be useful therapeutically against other diseases, particularly, virally or otherwise infected cells or tissues.

"Stress protein"("SP", "hsp") refers to any member of the various families of heat shock proteins. These families include, but are not limited to, hsp26, hsp40, hsp60, hsp70, hsp90, and hsp100. Preferably, the SPs are hsp72, hsp85 and hsp96. Most preferably the SP is hsp72.

2. Isolation of SPPCs, SPPC peptide and C-antigen

Exemplary methods for isolating SPPCs in general and C-antigen in particular follow. It is understood that the isolation methods can be modified by the addition or deletion of steps and changes in the steps within functional parameters. Provided the result of the isolation of at least one SPPC as defined and described herein and as detected or measured as described herein, the isolation method is encompassed by the invention. C-antigen is best characterized by obtaining the antigen following such a procedure and, particularly, the procedures more specifically provided in the Examples.

By way of example, tumor-associated SPPC can be purified to suitable homogeneity with the following protocol:

Tumor cells grown in tissue culture had their membranes disrupted, and an extract made by freeze-thaw. In particular detail, after cell harvest, cells are centrifuged at 1500 rpm for 10 min. The cells are washed twice in a PBS/1 mM phenylmethyl-sulfonyl fluoride (PMSF)/10μg/ml aprotinin solution. After washing, the pellet is resuspended in the wash solution and the cell concentration is adjusted to 10-20 x 10^6 cells/mL. This suspension is then subjected to five freeze-thaw sequences consisting of freezing in a dry-ice-acetone solution, followed immediately by thawing in a 37°C water bath. After the freeze-thaw treatments, the extract mixture is centrifuged at 1000 rpm to obtain a pellet of cellular debris and a supernatant.

The supernatant is combined with 3M ammonium sulfate buffer in a 2:1 ratio. This sample is then loaded onto a general-purpose hydrophobic chromatographic medium (preferably Phenyl Sepharose) at a rate of 0.5 ml/min using a pump. The column is connected to an FPLC system. Once loaded, the column is washed with 15 column volumes (CV) of Buffer A (50 mM sodium phosphate and 1M ammonium sulfate pH 7.0). The bound proteins are eluted step-wise with Buffer B (50 mM sodium phosphate pH 7.0). Active fractions are determined by immunological methods. During elution, the bulk of the bound proteins are eluted with 30% Buffer A/70% Buffer B. The 70% Buffer B elution is followed by 100% Buffer B. SPPC is eluted in the latter fraction. The positive fraction is concentrated on a membrane concentrator with a MW cut-off of 10 kD, preferably a Centriprep 10. The concentrated sample is passed

through a buffer exchange medium (preferably G-25) to the ADP-agarose chromatographic Buffer A (20 mM Tris-acetate, 20 mM NaCl, 3 mM $MgCl_2$, pH 7.5).

Six mL of the buffer exchanged material is incubated overnight with an additional 4 mL Buffer A and 5 mL ADP-agarose at 4°C on a platform shaker. Following incubation, the mixture is poured into a XK16 x 40 column. The column is washed with the ADP-agarose chromatographic Buffer A until the OD at 280 reaches baseline. The column is further washed with 0.5M NaCl in chromatographic-Buffer A and re-equilibrated with Buffer A. The bound protein is then eluted with 3 mM ADP in the ADP Buffer A and fractions collected. The active fraction is concentrated on a membrane concentrator with a MW cut-off of 10 kD (preferably Amicon).

The concentrated, eluted sample is diluted with anionic chromatographic Buffer A (20 mM Tris pH 7.8) at 1:10. One mL of diluted sample is loaded onto a strong anionic column (preferably a Mono Q Sepharose) attached to an FPLC. The flow rate is set at 1 ml/min. Fractions are collected and the antigenic fraction identified as outlined above. This three-step procedure gives a suitable, substantially homogeneous, active, SPPC.

ADP chromatographic media are media to which ADP is bound, and includes, but is not limited to, ADP bound to Sepharose and agarose. Preferably the medium is ADP agarose. Although the preceding method applies most aptly to hsp70 (particularly as detailed below) and (with limited routine modification, if any such modification is required) to hsp60, it can be used for tumor-associated SPPCs that are determined to be of the hsp20-30 and hsp40 families (with necessary modifications according to routine skill in the art). Additionally, in the case of the hsp90 family, a lectin column, preferably a Concanavalin A column, can substitute for the ADP chromatographic media described above.

Optionally, in a preferred method, C-antigen and other such SPPCs can be further purified under non-denaturing conditions, preferably in an electrophoretic extraction step. For example, after final concentration from the anionic column (particularly, in the case of C-antigen, the SPPC is already substantially purified), 15μL of the complex is mixed 50/50 with 2X Laemmli's buffer. The sample is separated on a suitable polyacrylamide gel electrophoresis apparatus under native, non-denaturing conditions (no SDS, mercaptoethanol or boiling). After completion of electrophoresis, the gel is blotted onto a membrane (PVDF or nitrocellulose) again under non-denaturing conditions. Identification of the SPPC location on the blotted membrane is

confirmed by treatment with an anti-SPPC antigen-binding fragment followed by binding an appropriately labeled secondary antibody. The SPPC can be "cut" from the membrane and the bound SPPC can be treated to cause the release of the peptide from the complex and subjected to further analysis. For example; the membrane can be treated to cause the release of the complex and the subsequently released peptide can be analyzed, for instance, by capillary electrophoresis and sequentially applied to a MALDI mass spectrograph.

An alternate method for the purification of SPs is developed from the creation of affinity chromatographic media of SPPC specific IgG antibodies or fragments thereof, for example the recombinant H11 IgG described in WO 97/44461. A 5 mL sample from a hydrophobic column (preferably Phenyl Sepharose) is incubated with 2 mL of SPPC-specific IgG Sepharose. The IgG-Sepharose/sample is incubated over-night at 4°C on a rotary shaker. After incubation, the mixture is poured into a small chromatographic column (preferably BioRad 10 mL Econo-Column). The column is washed with ten column volumes (CV) of PBS (pH 7.4) followed by three CV of 0.5 M NaCl in PBS. The affinity column is then re-equilibrated with PBS. Following equilibration, SPPC is eluted using a glycine buffer pH 2.8. The eluate is concentrated on a micro-pore concentrator (preferably Centriprep 3). The acid elution results in the dissociation of the SP from its peptide. The small molecular weight fraction (peptide) is concentrated with a peptide concentrator (preferably Microcon SCX). The purified SP is retained on the micro-pore concentrator.

After concentration, the eluted mixture of peptide and SP dissociated complex is passed through a peptide concentrator (preferably Microcon-SCX). The resultant material is freeze dried, and re-dissolved in 0.1% TFA. After re-constitution the material is fractionated on a reverse-phase HPLC column. Fractions are analyzed directly on a MALDI mass spectrometer.

Reconstitution of the peptide with the SP can be effected by any method known in the art such as mixing the affinity column purified SP with the peptide (purified native, recombinant or synthesized peptide) in PBS in the presence of 1 mM ADP and 1 mM $MgCl_2$ and incubating at 37°C for 30 min. Other suitable methods are described for instance in Davis et al. (1999) Proc. Natl. Acad. Sci. USA 96:9296.

3. <u>Isolation of SPPCs using an IgG Affinity Column Using Alkaline Elution Buffer</u>

100 mL of A-375 cell extract is centrifuged at 1400g for 30 min and the supernatant collected. The supernatant is then diluted five times with HiTrap Q buffer A (Tris 20 mM, pH

08/12/1999 17:50 416 362 0823 received

CA 02290722 1999-12-08

8.2). Diisopropyl fluorophosphate (DFP), a protease inhibitor, is added to a final concentration of 1 mM. The sample is loaded on a 10 mL anionic HiTrap Q column at a rate of 3 SP mL/min. The column is then washed with 15 CV of 50 SP mM NaCl in HiTrap Q buffer A. Bound protein is eluted with 20 CV of a 50 mM to 600 SP mM NaCl gradient and 10 SP mL fractions collected. Fractions are concentrated using Centriprep 10. The SPPC-containing fractions are identified by Western blot analysis using H11 IgG as the primary antibody and an appropriately labeled second antibody.

SPPC, partially purified through the HiTrap Q anionic column, is applied to the IgG affinity column and incubated for 2 hours at room temperature with gentle rotation. Following incubation, the column is washed with 20 SP mL TBS (Tris 20 SP mM, NaCl 150 mM, pH 7.4). The bound SPPC is eluted with 50 SP mM diethylamine pH 11. The eluted material is concentrated and the purity determined using Western blot analysis. The results show that, under these conditions, the SPPC is eluted intact.

4. Hsp96-specific Purification

In lieu of the ADP agarose purification step, hsp96 (Grp96) complexes can be purified as described by Blahere et al. *J. Exp. Med.* (1997) 186:1315-1322. Cancer cell extract is applied to a lectin column, specifically concanavalin A and incubated over-night at 4°C. The SP is eluted from the column with 10% α-methylmannoside. The hsp90 active fractions are concentrated on a micro-pore filter (preferably Centriprep 10).

SPPCs can be isolated from other diseased cells in a manner analogous to the methods described herein for isolation of such complexes from tumor cell extracts, for example cells which are virally or otherwise infected, with the result that the screening protocols described herein for differentiating between tumor and non-tumor cells could be analogously applied, by persons skilled in the art, according to methods within the skill of those in the art, to identify: 1) complexes that are found on the surface of infected but not tumor cells; and 2) antibodies which react specifically with such complexes.

5. Isolated Peptide

The invention further encompasses compositions comprising the isolated, disassociated SPPC peptides of the invention and functionally equivalent fragments and derivatives thereof. In the case of C-antigen, the invention encompasses peptides containing at least 5-10 amino acid residues of the peptide sequence.

B. SPPC - specific antigen-binding fragments.

1. Antigen-binding fragment and compositions thereof

This invention encompasses antigen-binding fragments that specifically recognize SPPCs in a tumor-or disease-associated manner. That is, in the case of tumors, the SPPC is predominantly found on tumor cells such that antigen-binding fragments that recognize the complexes preferentially recognize or bind to cancer cells. The term "disease-associated" means associated with cancer as well as one or more other pathologic conditions that induce cell surface expression of SPPC.

The invention further encompasses a composition of matter comprising an isolated antigen-binding fragment of an antibody which binds specifically to at least one SPPC which is specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer. In particular, the at least one SPPC contains a non-covalently bound peptide which confers the specific immunogenicity.

The invention further encompasses a composition comprising an antigen-binding fragment of an antibody which binds specifically to a plurality of SPPCs which is specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer. In particular, the SPPCs contain different non-covalently bound peptides, which confer the specific immunogenicity.

The term "antigen-binding fragment" includes any peptide that binds to the cancer-specific SPPCs in a cancer cell-specific manner. Typically, these fragments include such immunoglobulin fragments as Fab, $F(ab')_2$, Fab', scFv (both monomer and polymeric forms) and isolated H and L chains. An antigen-binding fragment retains specificity of the intact immunoglobulin, although avidity and/or affinity can be altered. First generation therapies are those based on such compounds and compositions. Especially preferred are the anti-C and anti-SPPC scFvs.

"H11" the exemplary anti-SPPC antibody is an antibody obtained from the fusion of peripheral blood lymphocytes of a 64 year old male with a low grade glioma and fused to a human myeloma cell line to produce a hybridoma designated NBGM1/H11. The generation and characterization of H11 is described in Example 1. "Anti-C" represents any antibody, or antigen-binding fragment thereof, either monoclonal, polyclonal or derivative thereof that recognizes

specifically the C-antigen and distinguishes between cancer and noncancer cells. Anti-C, as defined herein, does not include H11 and its derivatives.

Certain compounds, compositions and methods described in this application relate generally to anti-C and derivatives thereof which can be generated routinely by standard immunochemical techniques. This includes, but is not limited to, anti-C coupled to another compound by chemical conjugation, or associated with by mixing with an excipient or an adjuvant. Specific conjugation partners and methods of making them are described herein and well known in the art. More preferred are anti-C and anti-SPPC scFvs that are not coupled to a chemical agent.

Antigen-binding fragments (also encompassing "derivatives" thereof) are typically generated by genetic engineering, although they can be obtained alternatively by other methods and combinations of methods. This classification includes, but is not limited to, engineered peptide fragments and fusion peptides. Preferred compounds include polypeptide fragments containing the anti-SP-peptide CDRs, antibody fusion proteins containing cytokine effector components, antibody fusion proteins containing adjuvants or drugs, and, single-chain V region proteins. Antigen-binding fragments are considered to be of human origin if they are isolated from a human source, and used directly or cloned (either intact genes or portions thereof) and expressed in other cell types and derivatives thereof.

A "fusion polypeptide" is a polypeptide comprising contiguous peptide regions in a different position than would be found in nature. The regions can normally exist in separate proteins and are brought together in the fusion polypeptide; they can normally exist in the same protein but are placed in a new arrangement in the fusion polypeptide; or they can be synthetically arranged. For instance, as described below, the invention encompasses recombinant proteins (and the polynucleotides encoding the proteins or complementary thereto) that are comprised of a functional portion of an antigen-binding fragment and a toxin. Methods of making these fusion proteins are known in the art and are described for instance in WO93/07286.

A "functionally equivalent fragment" of a polypeptide varies from the native sequence by any combination of additions, deletions, or substitutions while preserving at least one functional property of the fragment relevant to the context in which it is being used. The antigen-binding fragments are designated anti-SP-peptide.

CA 02290722 1999-12-08

The antigen-binding fragments provided herein are useful in palliating the clinical conditions related to a wide variety of cancers. The invention encompasses antigen-binding fragments (excluding H11) recognizing C-antigen. These are designated anti-C. The invention further comprises polypeptide derivatives of the antigen-binding fragments and methods for using these compositions in diagnosis, treatment, and manufacture of novel reagents.

The invention also encompasses antigen-binding fragments conjugated to a chemically functional moiety. Typically, the moiety is a label capable of producing a detectable signal. These conjugated antigen-binding fragments are useful, for example, in detection systems such as quantitation of tumor burden, and imaging of metastatic foci and tumor imaging. Such labels are known in the art and include, but are not limited to, radioisotopes, enzymes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds, substrate cofactors and inhibitors. For examples of patents teaching the use of such labels, see, for instance U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. The moieties can be covalently linked, recombinantly linked, or conjugated (covalently or non-covalently) through a secondary reagent, such as a second antibody, protein A, or a biotin-avidin complex.

Other functional moieties include signal peptides, agents that enhance immunologic reactivity, agents that facilitate coupling to a solid support, vaccine carriers, bioresponse modifiers, paramagnetic labels and drugs. Signal peptides are described above and include prokaryotic and eukaryotic forms. Agents that enhance immunologic reactivity include, but are not limited to, bacterial superantigens and adjuvants. Agents that facilitate coupling to a solid support include, but are not limited to, biotin, avidin or derivatives thereof. Immunogen carriers include, but are not limited to, physiologically acceptable buffers. Bioresponse modifiers include, but are not limited to, cytokines, e.g. tumor necrosis factor (TNF), IL-2, interleukin-4 (IL-4), GM-CSF; and certain interferons. See also, US Patent 5,750,119; and WO patent publications: 96/10411; 98/34641; 98/23735; 98/34642; 97/10000; 97/10001; and 97/06821.

A "signal peptide" or "leader sequence" is a short amino acid sequence that directs a newly synthesized protein through a cellular membrane, usually the endoplasmic reticulum in eukaryotic cells, and either the inner membrane or both inner and outer membranes of bacteria. Signal peptides are typically at the *N*-terminus of a polypeptide and are removed enzymatically

between biosynthesis and secretion of the polypeptide from the cell. Thus, the signal peptide is not present in the secreted protein but is present only during protein production.

Suitable drug moieties include antineoplastic agents. These include, but are not limited to, radioisotopes, immunotoxins, vinca alkaloids such as the vinblastine, vincristine and vindesine sulfates, adriamycin, bleomycin sulfate, carboplatin, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, duanorubicin hydrochloride, doxorubicin hydrochloride, etoposide, fluorouracil, lomustine, mechloroethamine hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mitotane, pentostatin, pipobroman, procarbaze hydrochloride, streptozotocin, taxol and analogs thereof, thioguanine, and uracil mustard.

Immunotoxins, including single chain conjugates, can be produced by recombinant means. Production of various immunotoxins is well known in the art, and methods can be found, for example, in "Monoclonal Antibody-toxin Conjugates: Aiming the Magic Bullet," Thorpe et al. (1982) *Monoclonal Antibodies in Clinical Medicine*, Academic Press, pp. 168–190; Vitatta (1987) *Science* 238:1098–1104; and Winter and Milstein (1991) *Nature* 349:293–299. Suitable toxins include, but are not limited to, ricin, radionuclides, pokeweed antiviral protein, Pseudomonas exotoxin A, diphtheria toxin, ricin A chain, fungal toxins such as fungal ribosome inactivating proteins such as gelonin, restrictocin and phospholipase enzymes. See, generally, "Chimeric Toxins," Olsnes and Pihl, *Pharmac. Ther*. 15:355–381 (1981); and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159–179, 224–266, Academic Press (1985).

The chemically functional moieties can be made recombinantly for instance by creating a fusion gene encoding the antigen-binding fragment and functional regions from other genes (e.g. enzymes). In the case of gene fusions, the two components are present within the same gene. Alternatively, antigen-binding fragments can be chemically bonded to the moiety by any of a variety of well known chemical procedures. For example, when the moiety is a protein, the linkage can be by way of hetero-bifunctional cross linkers, e.g., SPDP, carbodiimide glutaraldehyde, or the like. The moieties can be covalently linked, or conjugated, through a secondary reagent, including, but not limited to a second antibody, protein A, or a biotin-avidin complex. Paramagnetic moieties and the conjugation thereof to antibodies are well-known in the art. See, e.g., Miltenyi et al. (1990) *Cytometry* 11:231-238.

Methods of antibody production and isolation are well known in the art. See, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, New York. The H11 antibody is a human immunoglobulin of the IgM subclass, and can be isolated by any technique suitable for immunoglobulins of this isotype such as by salt fractionation coupled with size exclusion to obtain a crude isolate. Antibody purification methods include, but are not limited to, salt precipitation (for example, with ammonium sulfate); ion exchange chromatography (for example, on a cationic or anionic exchange column run at neutral pH and eluted with step gradients of increasing ionic strength); gel filtration chromatography (including gel filtration HPLC); and chromatography on affinity resins such as protein A, protein G, hydroxyapatite, and anti-immunoglobulin. Antigen-binding fragments can also be purified on affinity columns comprising the C-antigen or an antigenic portion thereof. Preferably fragments are purified using Protein-A-CL-Sepharose™ 4B chromatography followed by chromatography on a DEAE-Sepharose™ 4B ion exchange column.

The invention also encompasses hybrid antibodies, for instance in which one pair of H and L chains is obtained from a first antibody, while the other pair of H and L chains is obtained from a different second antibody. For purposes of this invention, one pair of L and H chains is from anti-SP-peptide. In one example, each L-H chain pair binds different epitopes of the C-antigen. Such hybrids can also be formed using humanized H or L chains. The invention also encompasses other bispecific antibodies such as those containing two separate antibodies covalently linked through their constant regions.

Other antigen-binding fragments encompassed by this invention are antibodies in which the H or L chain has been modified to provide additional properties. For instance, a change in amino acid sequence can result in reduced immunogenicity of the resultant polypeptide. The changes range from changing one or more amino acids to the complete redesign of a region such as a C region domain. Typical changes include, but are not limited to, those related to complement fixation, interaction with membrane receptors, and other effector functions. A recombinant antibody can also be designed to aid the specific delivery of a substance (such as a cytokine) to a tumor cell. Also encompassed by the invention are peptides in which various immunoglobulin domains have been placed in an order other than that which occurs in nature.

The size of the antigen-binding fragments can be only the minimum size required to provide a desired function. It can optionally comprise additional amino acid sequence, either

native to the antigen-binding fragment, or from a heterologous source, as desired. Anti-SPPCs can contain only 5 consecutive amino acids from an anti-SP-peptide V region sequence. Polypeptides comprising 7 amino acids, more preferably about 10 amino acids, more preferably about 15 amino acids, more preferably about 25 amino acids, more preferably about 50 amino acids, more preferably about 75 amino acids from the anti-SP-peptide L or H chain V region are also included. Even more preferred are polypeptides, comprising the entire anti-SP-peptide L or H chain V region.

Substitutions can range from changing or modifying one or more amino acid residue to complete redesign of a region, such as the V region. Amino acid substitutions, if present, are preferably conservative substitutions that do not deleteriously affect folding or functional properties of the peptide. Groups of functionally related amino acids within which conservative substitutions can be made are glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine/methionine; lysine/arginine; and phenylalanine/tryosine/tryptophan. Antigen-binding fragments of this invention can be in glycosylated or unglycosylated form, can be modified post-translationally (e.g., acetylation, and phosphorylation) or can be modified synthetically (e.g., the attachment of a labeling group).

Polypeptide derivatives comprising both an L chain and an H chain can be formed as separate L and H chains and then assembled, or assembled in situ by an expression system for both chains. Such expression systems can be created by transfecting with a plasmid comprising separate transcribable regions for the L and H chain, or by co-transfecting the same cell with plasmids for each chain. In a third method, a suitable plasmid with an H chain encoding region is transfected into an H chain loss mutant.

H chain loss mutants can be obtained by treating anti-SP-peptide producing cells with fluorescein-labeled rabbit anti-mouse IgG (H chain specific, DAKO Corporation, Carpinteria, CA) according to the supplier's instruction. The stained and unstained cell populations are analyzed by flow cytometry. Unstained cells are collected in a sterilized tube and placed in 96-well plates at 1 cell/well by limiting dilution. Culture supernatants are then assayed by ELISA using goat anti-mouse IgG (H chain specific) and goat anti-mouse kappa. Clones having a kappa-positive, IgG-negative phenotype are subcloned at least 3 times to obtain stable anti-SP-peptide$^{(-H)}$ mutants. mRNA from putative H chain loss mutants can be isolated and the sequence of the L chain V region cDNA determined. Reverse PCR of the mRNA for the V_H is performed

with 2 sets of 5'- and 3'- primers, and used for cloning of anti-SP-peptide$^{(-H)}$ cDNA. An H chain loss mutant yields no detectable DNA band. Transfection of the cells proceeds with a suitable H chain plasmid.

Another antigen-binding fragment derivative encompassed by this invention is an antibody in which the constant region of the H or L chain has been modified to provide additional properties. For instance, a change in amino acid sequence can result in altered immunogenicity of the resultant polypeptide. The changes range from one or more amino acids to the complete redesign of constant region domain. Changes contemplated affect complement fixation, interaction with membrane receptors, and other effector functions. A recombinant antibody can also be designed to aid the specific delivery of a substance (such as a lymphokine) to an effector cell. Also encompassed by the invention are proteins in which various immunoglobulin domains have been placed in an order other than that which occurs in nature.

The invention also encompasses single chain V region fragments ("scFv") of anti-SP-peptides. Single chain V region fragments are made by linking L and/or H chain V regions by using a short linking peptide. Bird et al. (1988) *Science* 242:423-426. Any peptide having sufficient flexibility and length can be used as a linker in a scFv. Usually the linker is selected to have little to no immunogenicity. An example of a linking peptide is $(GGGGS)_3$, which bridges approximately 3.5 nm between the carboxy terminus of one V region and the amino terminus of another V region. Other linker sequences can also be used, and can provide additional functions, such as a means for attaching to a drug or solid support. Preferably, for therapeutic use, the scFvs are not coupled to a chemically functional moiety.

All or any portion of the H or L chain can be used in any combination. Typically, the entire V regions are included in the scFv. For instance, the L chain V region can be linked to the H chain V region. Alternatively, a portion of the L chain V region can be linked to the H chain V region, or portion thereof. Also contemplated are scFvs in which the H chain V region is from H11, and the L chain V region is from another immunoglobulin. It is also possible to construct a biphasic, scFv in which one component is an antigen-binding fragment and another component is a different polypeptide, such as a T cell epitope.

The scFvs can be assembled in any order, for example, V_H—(linker)—V_L or V_L—(linker)—V_H. There can be a difference in the level of expression of these two configurations in particular expression systems, in which case one of these forms can be

preferred. Tandem scFvs can also be made, such as (X)—(linker)—(X)—(linker)—(X), in which X are scFvs , or combinations thereof with other polypeptides. In another embodiment, single chain antibody polypeptides have no linker polypeptide, or just a short, inflexible linker. Possible configurations are V_L—V_H and V_H—V_L. The linkage is too short to permit interaction between V_L and V_H within the chain, and the chains form homodimers with a V_L/V_H antigen-binding site at each end. Such molecules are referred to in the art as "diabodies".

ScFvs can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid-containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as *Escherichia coli*, and the expressed protein can be isolated using standard protein purification techniques. ScFv can also be obtained from a phage display library as described in more detail herein and in Example 10.

A particularly useful system for the production of scFvs is plasmid pET-22b(+) (Novagen, Madison, WI) in *E. coli* pET-22b(+) contains a nickel ion binding domain consisting of 6 sequential histidine residues, which allows the expressed protein to be purified on a suitable affinity resin. Another example of a suitable vector is pcDNA3 (Invitrogen, San Diego, CA), described above.

Conditions of gene expression should ensure that the scFv assumes optimal tertiary structure. Depending on the plasmid used (especially the activity of the promoter), and the host cell, it can be necessary to modulate the rate of production. For instance, use of a weaker promoter, or expression at lower temperatures, can be necessary to optimize production of properly folded scFv in prokaryotic systems; or, it can be preferably to express scFv in eukaryotic cells.

The invention also encompasses polymeric forms of antigen-binding fragments, containing a plurality of anti-SP-peptide. One embodiment is a linear polymer of antigen-binding fragments, optionally conjugated to carrier. These linear polymers can comprise multiple copies of a single antigen-binding fragment polypeptide, or combinations of different polypeptides, and can have tandem polypeptides, or polypeptides separated by other amino acid sequences. Another embodiment is multiple antigen peptides (MAPs). MAPs have a small immunologically inert core having radially branching lysine dendrites, onto which a number of

antigen-binding fragment polypeptides are covalently attached. See for instance, Posnett et al. (1988) *J. Biol. Chem.* 263:1719-1725; and Tam (1989) *Met. Enz.* 168:7-15. The result is a large macromolecule having a high molar ratio of antigen-binding fragment polypeptides to core. MAPs are efficient immunogens and useful antigens for immunoassays. The core for creating MAPs can be made by standard peptide synthesis techniques, or obtained commercially (Quality Controlled Biochemicals, Inc., Hopkinton, MA). A typical core matrix is made up of three levels of lysine and eight amino acids.

The invention further includes anti-idiotypic-antigen-binding fragments to anti-C-antigen-specific antibodies. Such anti-idiotypes can be made by any method known in the art. Specifically, the invention encompasses anti-H11 anti-idiotype antigen-binding fragments. Anti-idiotypes are particularly suitable for use as vaccines.

Cancer patients are often immunosuppressed and tolerant to some tumor-associated antigens (TAA). Triggering an active immune response to such TAA represents an important challenge in cancer therapy. Immunization with a given antigen generates the production of antibodies against the antigen. The invention encompasses anti-tumor monoclonal antibodies; anti-idiotypic monoclonal antibodies; and anti-anti-idiotypic monoclonal antibodies. See also, PCT/US95/17103.

While vaccines are generally designed for asymptomatic individuals, vaccines can also be used to treat those with advanced cases of disease. For example, a vaccine therapy of 16 patients with advanced epithelial ovarian cancer or recurrences involved ACA125. ACA125 is an immunoglobulin G1 (IgG1) murine monoclonal anti-idiotype antibody that mimics a specific epitope on CA125, an antigen that is expressed by most malignant ovarian tumors. Nine of 16 patients developed a CA125-specific cellular immune response by their peripheral blood lymphocytes (PBL). Wagner et al. (1997) *Hybridoma* 16:33-40. For work related to the use of anti-idiotype antibodies in cancer vaccines, see Durrant et al. (1997) *Hybridoma* 16: 23-6.

Any carrier can be used which is not harmful to the host. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins; polysaccharides (such as latex functionalized Sepharose, agarose, cellulose, cellulose beads and the like); polymeric amino acids (such as polyglutamic acid, polylysine, and the like); amino acid copolymers; and inactive virus particles or attenuated bacteria, such as *Salmonella*. Especially useful carrier proteins are

serum albumins, keyhole limpet hemacyanin (KLH), certain Ig molecules, thyroglobulin, ovalbumin, and tetanus toxoid. KLH is especially preferred.

2. Methods of Obtaining anti-SPPCs

The invention encompasses methods of obtaining anti-SPPCs. Anti-SPPCs can be obtained and isolated in a number of ways.

Methods of generating new antigen-binding fragments to C-antigen or other such tumor-associated SPPCs, as detailed below, include: 1) employing phage display techniques (see, generally, Hoogenboom et al. (1998) *Immunotechnology* 4:1-20) by which cDNA encoding antibody repertoires are preferably amplified from lymphocyte or spleen RNA using PCR and oligonucleotide primers specific for species-specific V regions; 2) immunizing mammals with the antigen and generating polyclonal or monoclonal antibodies (Mabs); 3) generating hybridomas from cancer patients including human:human hybridomas; and 4) employing phage display to make antibodies without prior immunization by displaying on phage, very large and diverse V gene repertoires. Preferably, for therapeutic purposes, if non-human antibodies are to be used, can be humanized by any method known in the art.

With respect to the first of these techniques, the method of Medez et al. (1997) *Nature Genetics* 18:410 can be used. Briefly, purified SPPC (such as C-antigen), is used to immunize transgenic mice lacking the native murine antibody repertoire and instead having most of the human antibody V-genes in the germ line configuration. Human antibodies are subsequently produced by the murine B cells. The antibody genes are recovered from the B cells by PCR library selection or classic hybridoma technology.

Alternatively, by using the second of these techniques, antibodies can be obtained from mice (such as BALB/c) after injection with purified SP-peptide. Mabs are generated using standard hybridoma technology. See for instance, Maiti et al. (1997) *Biotechnology International* 1:85-93 (human hybridomas); and Kohler and Milstein (1975) *Nature* 256:495 – 497 (mouse hybridomas). Murine antibodies can be subsequently humanized for instance by the method of Rosok et al. (1996) *J. Biol. Chem.* 271:22611-22618; Baca et al. (1997) *J. Biol. Chem.* 272:10678-10684; Rader et al. *Proc. Natl. Acad. Sci. USA* 95:8910-8915; and Winter and Milstein (1991) *Nature* 349:293-299.

According to the third technique, a phage display approach can be used to rapidly generate human antibody against C-antigen or other SPPCs. This approach can employ the

CA 02290722 1999-12-08

method outlined by Henderikx et al. (1998) *Cancer Res.* 58:4324-32. Antibody fragments displayed on phage are selected from a large naïve phage antibody/fragment library containing different single chain antibodies by separating those which bind to immobilized antigen. As regards the antigen, preferably the entire SPPC is used. Human antibody fragments are selected from naïve repertoires constructed either from germline V-domains or synthesized with many mutations (mutations are targeted either by homologous gene re-assortments or error prone PCR) in both the framework and CDR regions. Antigen-binding fragments specifically reactive with SPPCs can be screened against tumor and normal tissues as described herein in order to identify tumor-specific antigen-binding fragments.

The invention also encompasses methods of identifying antigen-binding fragments specific for a tumor-associated SPPCs by generating a suitable phage display library; isolating SPPCs from a tumor or recombinant host; screening the phage display library with the complexes according to standard immunochemical techniques to obtain phage that display an antigen-binding fragment that binds specifically to SPPC; and screening the phage obtained for specific cell surface tumor-associated reactivity, by screening against tumor and normal cells and selecting the phage that bind preferentially to tumor but not normal cells. Methods of generating antigen-binding fragments by phage display are well known in the art. See, Hoogenboom et al. (1998) *Immunotechnology* 4:1-20.

Lymphocyte (PBL) or spleen RNA is typically used to make antibody fragment repertoires. Mutagenesis using homologous reassortment or error prone PCR increases diversity.

Phage display libraries created from human lymphocytes of cancer patients are expected to be enriched in antibodies specific for tumor-associated SPPCs. Also, antibody phage display libraries have been prepared from B-cells of patients undergoing active specific immunotherapy (ASI) with autologous tumor cells. Hall et al. (1998) *Immunotechnology* 4:127-140.

Repertoires of antibody genes can be amplified from immunized mice or humans using PCR and scFv or Fab antibody fragments obtained thereby can be cloned and expressed on the surface of bacteriophage. The antibody gene repertoires are amplified from lymphocyte or spleen RNA using PCR and oligonucleotide primers specific for host animal-specific V regions. Phage display can also be used to make antibodies without prior immunization by displaying very large and diverse V gene repertoires on phage. The natural V gene repertoires present in PBL (peripheral blood lymphocytes) are isolated by PCR amplification and the VH and VL

CA 02290722 1999-12-08

regions are spliced together at random using PCR. Mutations can be targeted to the V-domain genes by homologous gene reassortments (Zhao et al. *Nat. Biotechnol.* (1998) 15:258) or error-prone PCR. Hoogenboom et al. *Immunotechnology* (1998) 4:1-20. Totally synthetic human libraries can also be created and used to screen for SPPC-specific antibody fragments. Regardless of the method used to operate the phage display library, each resulting phage has a functional antibody fragment displayed on its surface and contains the gene encoding the antibody fragment in the phage genome. See, e.g. WO97/02342.

Affinity chromatography in which binding antibodies can be subtracted from non-binding antibodies has been established for some time. Nissim et al. (1994) *EMBO J.* 13:692-698; and Vaughan et al. (1996) *Nat. Biotechnol.* 14:309-314. Critical parameters affecting success are the number and affinity of antibody fragments generated against a particular antigen. Until recently, the production of large, diverse libraries remained somewhat difficult. Historically, scFv repertoires have been assembled directly from VH and VL RT-PCR products. RNA availability and the efficiency of RT-PCR were limiting factors of the number of V genes available. Also, assembly was based on ligating three fragments, namely VH and VL and the linker regions. Marks et al. (1991) *J. Mol. Biol.* 222:581-597.

An improved library construction method (Sheets et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:6175-6162) uses cloned VH and VL gene repertoires in separate plasmid vectors to provide a stable and limitless supply of material for scFv assembly. Also, the efficiency is increased by having DNA encoding the linker region at the 5' end of the VL library. Therefore there are only two fragments to be ligated instead of three.

The improved strategies (Sheets et al.) for generating phage antibody libraries have been demonstrated to efficiently and rapidly produce high affinity antibodies to a wide variety of protein antigens. Thus, a large library ($> 6.0 \times 10^9$) of phage displayed antibody fragments (e.g. scFv), panned against SPPCs can result in the selection of a panel of high affinity antibodies. See, Example 10 for a method overview.

Anti-SPPCs can also be derived or manipulated using genetic recombination. For example, the immunogenic activity of the V regions of the L and H chains can be screened by preparing a series of short polypeptides that together span the entire V region amino acid sequence. Using a series of polypeptides of 20 or 50 amino acids in length, each V region can be surveyed for useful functional properties. It is also possible to carry out a computer analysis of a

protein sequence to identify potentially interesting polypeptides. Such peptides can then be synthesized and tested for immunogenic activity.

The invention further encompasses various adaptations of antigen-binding fragments described in this section combined in various fashions to yield other anti-SP-peptides with desirable properties. For instance, anti-SPPCs with modified residues can be comprised in a MAP. In another example, an anti-SPPC scFv is fused to a cytokine, such as IL-2. All such combinations are contemplated in this invention.

The antigen-binding fragments of this invention can be made by any suitable procedure, including proteolysis of the antibody, by recombinant methods or by chemical syntheses. These methods are known in the art and need not be described in detail herein. Examples of proteolytic enzymes include, but are not limited to, trypsin, chymotrypsin, pepsin, papain, V8 protease, subtilisin, plasmin, and thrombin. Intact anti-SPPCs can be incubated with one or more proteinases simultaneously or sequentially. Alternatively, or in addition, intact antibody can be treated with disulfide reducing agents. Peptides can then be separated from each other by techniques known in the art, including but not limited, to gel filtration chromatography, gel electrophoresis, and reverse-phase HPLC.

Anti-SPPCs can also be made by expression from a polynucleotide encoding the peptide, in a suitable expression system by any method known in the art. Typically, polynucleotides encoding a suitable polypeptide are ligated into an expression vector under control of a suitable promoter and used to genetically alter the intended host cell. Both eukaryotic and prokaryotic host systems can be used. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use. Examples of prokaryotic host cells appropriate for use with this invention include *E. coli*. Examples of eukaryotic host cells include avian, insect, plant, and animal cells such as COS7, HeLa, and CHO cells.

Optionally, matrix-coated channels or beads and cell co-cultures can be included to enhance growth of antibody-producing cells. For the production of large amounts of antibody, it is generally more convenient to obtain ascitic fluid. The method of raising ascites generally comprises injecting hybridoma cells into an immunologically naïve, histocompatible or immunotolerant mammal, especially a mouse. The mammal can be primed for ascites production by prior administration of a suitable composition; e.g., Pristane. The ascitic fluid is then removed from the animal and processed to isolate the antibodies.

CA 02290722 1999-12-08

Alternatively, antigen-binding fragments can be chemically synthesized using amino acid sequence data and other information provided in this disclosure, in conjunction with standard methods of protein synthesis. A suitable method is the solid-phase Merrifield technique. Automated peptide synthesizers are commercially available, such as those manufactured by Applied Biosystems, Inc. (Foster City, CA).

Another method of obtaining anti-SPPCs is to immunize suitable host animals with tumor- or disease-associated SPPCs and to follow standard procedures for polyclonal or Mab production and isolation. Mabs thus produced can be "humanized" by methods known in the art. The invention thus encompasses humanized Mabs.

"Humanized" antibodies are those in which at least part of the sequence has been altered from its initial form to render it more like human immunoglobulins. In one version, the H chain and L chain C regions are replaced with human sequence. This is a fusion polypeptide comprising an anti-SPPC V region and a heterologous immunoglobulin (C) region. In another version, the CDR regions comprise anti-SPPC amino acid sequences, while the V framework regions have also been converted to human sequences. EP 0329400. In a third version, V regions are humanized by designing consensus sequences of human and mouse V regions, and converting residues outside the CDRs that are different between the consensus sequences.

In making humanized antibodies, the choice of framework residues help in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen. Glaser et al. (1992) *J. Immunol.* 149:2606; Tempest et al. (1992) *Biotechnology* 9:266; and Shalaby et al. (1992) *J. Exp. Med.* 17:217. The more homologous a human antibody is to the original murine antibody, the less likely that the human framework will introduce distortions into the murine CDRs that could reduce affinity. Based on a sequence homology search against an antibody sequence database, the human antibody IC4 provides good framework homology to muM4TS.22, although other highly homologous human antibodies would be suitable as well, especially kappa L chains from human subgroup I or H chains from human subgroup III. Kabat et al. (1987). Various computer programs such as ENCAD (Levitt et al. (1983) *J. Mol. Biol.* 168:595) are available to predict the ideal sequence for the V region. The invention thus encompasses human antibodies with different V regions. It is within the skill of one in the art to determine suitable V region

CA 02290722 1999-12-08

sequences and to optimize these sequences. Methods for obtaining antibodies with reduced immunogenicity are also described in U.S. Patent No. 5,270,202 and EP 699,755.

In certain applications, such as when an antigen-binding-fragment is expressed in a suitable storage medium such as a plant seed, the antigen-binding-fragment can be stored without purification. Fiedler et al. (1995) *Biotechnology* 13:1090-1093. For most applications, it is generally preferable that the polypeptide is at least partially purified from other cellular constituents. Preferably, the antigen-binding fragment is at least about 50% pure as a weight percent of total protein. More preferably, the antigen-binding fragment is at least about 50-75% pure. For clinical use, the antigen-binding fragment is preferably at least about 80% pure.

If the compositions of the invention are to be administered to an individual, the antigen-binding fragment is preferably at least 80% pure, more preferably it is at least 90% pure, even more preferably it is at least 95% pure and free of pyrogens and other contaminants. In this context, the percent purity is calculated as a weight percent of the total protein content of the preparation, and does not include constituents which are deliberately added to the composition after the antigen-binding fragment is purified.

The invention also encompasses methods of detecting cancer or disease-associated SPPCs in a biological sample. The methods include obtaining a biological sample, contacting the sample with an anti-SPPC under conditions that allow antibody-antigen-binding and detecting binding, if any, of the antibody to the sample as compared to a control, non-cancerous or non-diseased biological sample.

The invention also encompasses methods of detecting anti-SPPCs in a biological sample. These methods are applicable in a clinical setting, for example, for monitoring antibody levels in an individual, as well as an industrial setting, as in commercial production of anti-SPPCs.

After a biological sample is suitably prepared, for instance by enriching for antibody concentration, it is mixed with excess anti-SPPC under conditions that permit formation of a complex between SPPC and any target antibody that can be present. The amount of complex is then determined, and compared with complexes formed with standard samples containing known amounts of target antibody in the range expected. Complex formation can be observed by immunoprecipitation or nephelometry, but it is generally more sensitive to employ a reagent labeled with such labels as radioisotopes like ^{125}I, enzymes like peroxidase and β-galactosidase, or fluorochromes like fluorescein.

Anti-SPPC can be characterized by any method known in the art. For instance, by the ability to bind specifically to tumors, cancer cell lines, C-antigen or a tumor- or disease-associated SPPC. An antigen-binding fragment can also be tested for the ability to specifically inhibit the binding between antigen and intact antibody either competitively or non-competitively. Anti-SPPCs can also be tested for their ability to palliate or ameliorate neoplastic disease, such as carcinomas. It is understood that only one of these properties need be present in order for a polypeptide to come within the scope of this invention, although preferably more than one of these properties is present.

The ability of an anti-SPPC to bind antigen can be tested by any immunoassay known in the art. Any form of direct binding assay is suitable. In one such assay, one of the binding partners, the antigen or the putative anti-SPPC, is labeled. Suitable labels include, but are not limited to, radioisotopes such as ^{125}I, enzymes such as peroxidase, fluorescent labels such as fluorescein, and chemiluminescent labels. Typically, the other binding partner is insolubilized (for example, by coating onto a solid phase such as a microtiter plate) to facilitate removal of unbound soluble binding partner. After combining the labeled binding partner with the unlabeled binding partner, the solid phase is washed and the amount of bound label is determined. Another such assay is a sandwich assay, in which the putative anti-SPPC is captured by a first anti-immunoglobulin on a solid phase, the anti-SPPC is added and any resultant captured complex is labeled and with an antibody that binds to anti-SPPC. The anti-immunoglobulin can be specific, for instance, an antibody constant region such as by mouse anti-human IgG. In either of these examples, the extent of binding of anti-SPPC is related to the amount of label bound to the solid phase.

When used for immunotherapy, anti-SPPCs can be unlabeled or labeled with a therapeutic agent as described herein and as known in the art. These agents can be coupled either directly or indirectly to the antigen-binding fragments of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener et al. (1986) *Science* 231:148) and can be selected to enable drug release at the target site. Examples of therapeutic agents that can be coupled to antigen-binding fragments for immunotherapy include, but are not limited to bioresponse modifiers, drugs, radioisotopes, lectins, and toxins. Bioresponse modifiers include lymphokines which include, but are not limited to, TNF-α, IL-1, -2, and 3, lymphotoxin, macrophage activating factor,

migration inhibition factor, colony stimulating factor, and IFs. Interferons with which antigen-binding fragments can be labeled include IFN-α, IFN-β, and IFN-γ and their subtypes.

In using radioisotopically conjugated antigen-binding fragments for immunotherapy, certain isotypes can be more preferable than others depending on such factors as leukocyte distribution as well as isotype stability and emission. If desired, the malignant cell distribution can be evaluated by the *in vivo* diagnostic techniques described below. Depending on the malignancy, some emitters are preferable. In general, alpha and beta particle-emitting radioisotopes are preferred in immunotherapy. For example, if a subject has solid tumor foci, as in a carcinoma, a high energy beta emitter capable of penetrating several millimeters of tissue, such as ^{90}Y, can be preferable. On the other hand, if the malignancy consists of simple target cells, as in the case of leukemia, a short range, high energy alpha emitter, such as ^{212}Bi, can be preferable. Examples of radioisotopes which can be bound to the antigen-binding fragments of the invention for therapeutic purposes include, but are not limited to, ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, and ^{188}Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is preferably accomplished by binding the alpha-peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an alpha and beta subunit which under proper conditions can be separated. The toxic A chain component can be bound to an anti-SPPC and used for site specific delivery to a neoplastic cell.

Thus, for example, anti-SP-peptide can be used in combination with IFN-α. This treatment modality enhances Mab targeting of melanomas by increasing the expression of Mab reactive antigen by the melanoma cells. Greiner et al. (1987) *Science* 235:895. Alternatively, anti-SPPC can be used, for example, in combination with IFN-γ to thereby activate and increase the expression of Fc receptors by effector cells which, in turn, results in an enhanced binding of the antigen-binding fragments to the effector cell and killing of target malignant cells. Those of

skill in the art will be able to select from the various biological response modifiers to create a desired effector function that enhances the efficacy of anti-SPPC.

C. Polynucleotides

1. Compositions of Matter

A "polynucleotide" is a polymeric form of nucleotides of any length that contains deoxyribonucleotides, ribonucleotides, and analogs thereof in any combination. Polynucleotides can have any three-dimensional structure, and can perform any polynucleotide-specific function, known or unknown. The term "polynucleotide" includes double-, single-stranded, and triple-helical molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double stranded form of either the DNA, RNA or hybrid molecules.

A functionally equivalent fragment of a polynucleotide either encodes a polypeptide that is functionally equivalent to the original polypeptide when produced by an expression system, or has similar hybridization specificity as the original polynucleotide when used in a hybridization assay. A functionally equivalent fragment of a native antigen-binding fragment described herein typically has one or more of the following properties: ability to bind tumor- or disease-associated SPPCs; ability to bind at least one type of cancer cell in a specific manner; and an ability to elicit a cancer-specific immune response.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, viruses, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified , such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to other compounds or supports, including, without limitation, proteins, metal ions, labeling components, other polynucleotides, or a solid support.

The term "recombinant" polynucleotide means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin that either does not occur in nature or is covalently linked to another polynucleotide in an arrangement not found in nature. Recombinant methods are well known in the art. The practice of the invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (Gait, ed., 1984); "Animal Cell Culture" (Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (Wei & Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (Miller & Calos, eds., 1987); "Current Protocols in Molecular Biology" (Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (Coligan et al., eds., 1991). These techniques are applicable to the production of the polynucleotides and polypeptides, and, as such, can be considered in making and practicing the invention. Particularly useful techniques for particular embodiments are discussed in the sections that follow.

A "vector" refers to a recombinant plasmid or virus that comprises a heterologous polynucleotide to be delivered, either in vitro or in vivo, into a target cell. The heterologous polynucleotide can comprise a sequence of interest for purposes of therapy, and can be optionally in the form of an expression cassette. As used herein, a vector need not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors for the replication of a polynucleotide, and expression vectors for translation of a polynucleotide encoding sequence. Also included are viral vectors, which comprise a polynucleotide encapsidated or enveloped in a viral particle.

A "cell line" or "cell culture" denotes bacterial, plant, insect or higher eukaryotic cells grown or maintained in vitro. The progeny of a cell can not be completely identical (either morphologically, genotypically, or phenotypically) to the parent cell. A hybridoma refers to a cell line that produces a Mab. Methods of making hybridomas, both murine and human, are known in the art. Particular methods of producing human hybridomas are described and referenced throughout the specification.

A "host cell" denotes a prokaryotic or eukaryotic cell that has been genetically altered, or is capable of being genetically altered by administration of an exogenous polynucleotide, such as a recombinant plasmid or vector. When referring to genetically altered cells, the term refers both to the originally altered cell, and to the progeny thereof.

"Heterologous" refers to an entity genotypically distinct from the entity to which it is being compared. For example, a polynucleotide can be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence other than the native sequence is a heterologous promoter.

An "isolated" polynucleotide, polypeptide or SP-peptide complex is one that is substantially free of the materials with which it is associated in its native environment. By substantially free is meant that at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 95% free of these materials, and even more preferably to clinically acceptable standards of purity. The "native environment" is the cell in which it is synthesized whether in vitro or in vivo.

A "stable duplex" of polynucleotides refers to a duplex that is sufficiently long-lasting to persist between the formation of the duplex or complex and subsequent detection, including any optional washing steps or other manipulation that can take place in the interim.

The invention also encompasses polynucleotides encoding for functionally equivalent variants and derivatives of the native peptide and functionally equivalent fragments thereof which can enhance, decrease or not significantly affect properties of the polypeptides encoded thereby. These functionally equivalent variants, derivatives, and fragments display the ability to specifically recognize disease and tumor-associated SPPCs. For instance, changes in a DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect properties of the encoded polypeptide.

The polynucleotides of the invention can comprise additional sequences, such as additional encoding sequences within the same transcription unit, controlling elements such as promoters, ribosome binding sites, and polyadenylation sites, additional transcription units under control of the same or a different promoter, sequences that permit cloning, expression, and

transformation of a host cell, and any such construct as can be desirable to provide embodiments of this invention.

The invention encompasses a polynucleotide of at least about 9-15 consecutive nucleotides, preferably at least about 20 nucleotides, more preferably at least about 25 consecutive nucleotides, more preferably at least about 35 consecutive nucleotides, more preferably at least about 50 consecutive nucleotides, even more preferably at least about 75 nucleotides, still more preferably at least about 100 nucleotides, still more preferably at least about 200 nucleotides, and even more preferably at least about 300 nucleotides that forms a stable hybrid with a polynucleotide encoding the L chain or H chain V region of anti-SP-peptide, but not with other immunoglobulin encoding regions known at the time of filing of this application. Any set of conditions can be used for this test, as long as at least one set of conditions exist wherein the test polynucleotide demonstrates the required specificity.

Hybridization reactions can be performed under conditions of different "stringency." Conditions that increase stringency of a hybridization reaction are known. See, for example, Sambrook et al. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or deionized water.

The polynucleotides of this invention can be obtained using chemical synthesis, recombinant cloning methods, PCR, or any combination thereof. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequence data provided herein to obtain a desired polynucleotide by employing a DNA synthesizer or ordering from a commercial service.

Alternatively, nucleotides can be obtained from cell lines producing the peptide, cloning vectors, or expression vectors. RNA or DNA encoding the desired sequence can be isolated, amplified, and processed by standard recombinant techniques. Such techniques include digestion with restriction endonucleases, and amplification by polymerase chain reaction (PCR), or a suitable combination thereof. PCR technology is described in U.S. Patent Nos. 4,683,195,

4,800,159, 4,754,065 and 4,683,202, as well as *PCR: The Polymerase Chain Reaction*, Mullis et al. eds., Birkauswer Press, Boston (1994).

(a) SPPC peptide encoding polynucleotides.

The invention encompasses compositions comprising polynucleotides that encode SPPC peptides. In the case of C-antigen, the polynucleotides encode at least three consecutive amino acid residues an SPPC.

(b)Polynucleotides encoding antigen-binding fragments.

The invention encompasses polynucleotides encoding anti-C, derivatives thereof and complementary polynucleotides therefor. Methods of use of the polynucleotides are also encompassed by the invention. Methods of obtaining polynucleotides encoding anti-SPPC and methods of use thereof are the same as for anti-C. As used herein anti-SPPC encompasses anti-C. As used herein, anti-C and anti-SPPC specifically exclude H11 and derivatives thereof.

The invention further comprises polynucleotides encoding the SPPC-specific antibody V regions and derivatives thereof. These include isolated polynucleotide fragments, recombinant polynucleotides, and therapeutic plasmids and vectors, such as vaccinia vectors, comprising the polynucleotides.

Included in all these embodiments are polynucleotides with encoding regions for anti-SP-peptides, fusion proteins, humanized immunoglobulins, single-chain V regions, and other particular polypeptides of interest. These polypeptides are described above.

The invention also provides polynucleotides covalently linked with a detectable label. Such polynucleotides are useful, for example, as probes for detection of related nucleotide sequences.

2. Recombinant Expression Vectors.

Polynucleotides comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides can be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by any method known in the art, including, but not limited to, direct uptake, endocytosis, transfection, f-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. Amplified DNA can be isolated from the host cell by standard methods: see, e.g., Sambrook et al. (1989). RNA

can also be obtained from transformed host cell, or it can be obtained by using a DNA-dependent RNA polymerase.

The invention further includes a variety of vectors comprising a polynucleotide encoding anti-SPPC. These vectors can be used for expression of recombinant polypeptides as well as a source of anti-SPPC polynucleotides. Cloning vectors can be used to obtain replicate copies of the polynucleotides they contain, or as a means of storing the polynucleotides in a depository for future recovery. Expression vectors (and host cells containing these expression vectors) can be used to obtain polypeptides produced from the polynucleotides they contain. They can also be used where it is desirable to express anti-SPPC in an individual and thus have intact cells capable of synthesizing the polypeptide, such as in gene therapy. Suitable cloning and expression vectors include any known in the art, e.g., those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors and suitable host cells are known in the art and are not described in detail herein. See e.g. Gacesa and Ramji, (1994) *Vectors*, John Wiley & Sons.

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will grow under selective conditions. Typical selection genes either: (a) confer resistance to antibiotics or other toxic substances, e.g., ampicillin, neomycin, methotrexate; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from a defined medium. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art. Vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors can be constructed according to standard techniques, or can be selected from a large number of cloning vectors available in the art. While the cloning vector selected can vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, can possess a single target for a particular restriction endonuclease, or can carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28.

These and many other cloning vectors are available from commercial vendors such as BioRad, Stratagene, and Invitrogen.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide encoding an anti-SP-peptide of interest. The polynucleotide encoding the anti-SPPC is operatively linked to suitable transcriptional controlling elements, such as promoters, enhancers and terminators. For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons. These controlling elements (transcriptional and translational) can be derived from a gene encoding an anti-SPPC, or they can be heterologous (i.e., derived from other genes or other organisms). A polynucleotide sequence encoding a signal peptide can also be included to allow an anti-SPPC to cross or lodge in cell membranes or be secreted from the cell. A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are known in the art. One example of an expression vector is pcDNA3 (Invitrogen, San Diego, CA, in which transcription is driven by the cytomegalovirus (CMV) early promoter/enhancer. This vector also contains recognition sites for multiple restriction enzymes for insertion of the polynucleotide of interest. Another example of an expression vector (system) is the baculovirus/insect system.

Also encompassed herein are expression systems suitable for use in antibody-targeted gene therapy comprising a polynucleotide encoding an anti-SPPC. Suitable systems are described for instance by Brown et al. (1994) *Virol.* 198:477–488; and Miyamura et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8507–8511.

The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection. The choice of means of introducing vectors or polynucleotides encoding anti-SPPCs will often depend on features of the host cell.

Once introduced into a suitable host cell, expression of an anti-SPPC can be determined using any assay known in the art. For example, the presence thereof can be detected by RIA or ELISA of the culture supernatant (if the polypeptide is secreted) or cell lysates.

A vector of this invention can contain one or more polynucleotides encoding an anti-SPPC. It can also contain polynucleotide sequences encoding other polypeptides that enhance,

CA 02290722 1999-12-08

facilitate, or modulate the desired result, such as lymphokines, including, but not limited to, IL-2, IL-4, GM-CSF, TNF-α and IFN-γ. A preferred lymphokine is GM-CSF. Preferred GM-CSF constructs are those which have been deleted for the AU-rich elements from the 3' untranslated regions and sequences in the 5' untranslated region that are capable of forming a hairpin loop. Also embodied in this invention are vaccinia vectors encoding for recombinant anti-SPPCs, such as scFvs and other antigen-binding fragments, chimeras, and polymers. The invention further encompasses the generation of antigen-binding fragments from phage display libraries that have been selected by at least one round of screening with C-antigen or other disease- or cancer-associated SP-peptide. This includes use of phage display to humanize murine antibodies/antibody fragments to SPPCs. See, for example, (1996) *J. Biol. Chem.* 13:271; (1997) *J. Biol. Chem.* 18:272, and 10678-10684; and (1998) *Proc. Natl. Acad. Sci. USA* 95:8910-8915. Isolated phage and the anti-SPPCs encoded therein obtained by such a screening process are also included in the invention.

3. Host Cells.

Other embodiments of this invention encompass host cells transformed with polynucleotides encoding anti-SPPCs and vectors comprising anti-SPPCs polynucleotide sequences, as described above. Both prokaryotic and eukaryotic host cells can be used. Prokaryotic hosts include bacterial cells, for example *E. coli* and *mycobacteria*. Among eukaryotic hosts are yeast, insect, avian, plant and mammalian cells. Host systems are known in the art and need not be described in detail herein. Examples of a mammalian host cells include CHO and NS0, obtainable from the European Collection of Cell Cultures (England). Transfection of NS0 cells with a plasmid, for example, which is driven by a CMV promoter, followed by amplification of this plasmid in using glutamine synthetase provides a useful system for protein production. Cockett et al. (1990) *Bio/Technology* 8:662-667.

The host cells of this invention can be used, inter alia, as repositories of polynucleotides encoding anti-SPPCs, or as vehicles for production thereof. They can be used also as vehicles for in vivo expression of anti-SPPCs. The polynucleotides of this invention can be used in expression systems to produce polypeptides, intact antigen-binding fragments, or recombinant forms thereof.

4. Methods of use of the polynucleotides.

The polynucleotides of this invention have several uses. For example, in expression systems for the production of anti-SPPC. They are also useful as hybridization probes to assay for the presence of polynucleotides encoding anti-SPPC or related sequences in a sample using methods well known to those in the art. Further, the polynucleotides are also useful as primers to effect amplification of desired polynucleotides. The polynucleotides of this invention are also useful in pharmaceutical compositions including vaccines and for gene therapy.

The polynucleotides can also be used as hybridization probes for detection of anti-SPPC encoding sequences. Suitable hybridization samples include cells transformed ex vivo for use in gene therapy. In one illustration, DNA or RNA is extracted from a sample, and optionally run on a gel and/or digested with restriction endonucleases. The processed sample polynucleotide is typically transferred to a medium suitable for washing. The sample polynucleotide is then contacted with the anti-SPPC polynucleotide probe under conditions that permit a stable duplex to form if the sample contains a matching polynucleotide sequence. Any stable duplexes formed are detected by any suitable means. For example, the polynucleotide probe can be supplied in labeled form, and label remaining with the sample after washing will directly reflect the amount of stable duplex formed. In a second illustration, hybridization is performed in situ. A suitably prepared tissue sample is overlaid with a labeled probe to indicate the location anti-SPPC encoding sequences.

A short polynucleotide can also be used as a primer for a PCR reaction, particularly to amplify a longer sequence comprising a region hybridizing with the primer. This can be conducted preparatively, in order to produce polynucleotide for further genetic manipulation. It can also be conducted analytically, to determine whether an anti-SPPC encoding polynucleotide is present, for example, in a sample of diagnostic interest.

Another use of the polynucleotides is in vaccines and gene therapy. The general principle is to administer the polynucleotide so that it either promotes or attenuates the expression of the polypeptide encoded therein. Thus, the invention includes methods of inducing an immune response and methods of treatment comprising administration of an effective amount polynucleotides encoding anti-SPPC or an SPPC to an individual. In these methods, a polynucleotide encoding an anti-SPPC or SPPC is administered to an individual, either directly or via cells transfected with the polynucleotide. Preferably, the polynucleotide is in the form of a

CA 02290722 1999-12-08

circular plasmid, preferably in a supercoiled configuration. Preferably, the polynucleotide is replicated inside a cell. Thus, the polynucleotide is operatively linked to a suitable promoter, such as a heterologous promoter that is intrinsically active in cells of the target tissue type. Preferably, once in cell nuclei, plasmids persist as circular non-replicating episomal molecules. In vitro mutation can be carried out with plasmid constructs to encode, for example, molecules with greater affinity and/or avidity.

To determine whether plasmids containing polynucleotides encoding anti-SPPC are capable of expression in eukaryotic cells, cells such as COS-7, CHO, or HeLa can be transfected with the plasmids. Expression is then determined by immunoassay; for example, by Western blot. Smaller SPPCs can be detected, for example, by constructing the plasmid so that the resultant polypeptide is fused with a tag, such as a target epitope or enzyme label. Further characterization of the expressed polypeptide can be achieved by purifying the peptide and then conducting one of the functional assays described herein.

D. Kits

The invention encompasses kits containing anti-SPPC. Diagnostic procedures using the kits can be performed by diagnostic laboratories, experimental laboratories, practitioners, or private individuals. The clinical sample is optionally pre-treated for enrichment of the target being tested for. The user then applies a reagent contained in the kit in order to detect the changed level or alteration in the diagnostic component.

Each kit comprises antigen-binding fragments used for detecting cancer-associated SPPC in the sample. Optionally, the reagent can be conjugated with a label to permit detection of any complex formed with the target in the sample. In another option, a second reagent is provided that is capable of combining with the first reagent after it has found its target and thereby supplying the detectable label. For example, labeled anti-human IgG can be provided as a secondary reagent for use with intact anti-SP-peptide. Labeled avidin is a secondary reagent when the primary reagent has been conjugated to biotin.

The kits can be employed on a variety of biological samples including, both liquid samples cell suspensions and tissue samples. Suitable assays using anti-C that can be supplied in kit form include those described herein.

Each reagent is supplied in a solid form or dissolved/suspended in a liquid buffer suitable for inventory storage and later for exchange or addition into the reaction medium when the test is

CA 02290722 1999-12-08

performed. Suitable packaging is provided. The kit can optionally provide additional components that are useful in the procedure. These optional components include, but are not limited to, buffers, capture reagents, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

E. Therapeutic Compositions

1. Compositions of Matter.

The preparation of pharmaceutical compositions described herein is conducted in accordance with generally accepted procedures for the preparation of pharmaceutical preparations. See, for example, *Remington's Pharmaceutical Sciences 18th Edition* (1990), E.W. Martin ed., Mack Publishing Co., PA. Depending on the intended use and mode of administration, it can be desirable to process the active ingredient further in the preparation of pharmaceutical compositions. Appropriate processing can include sterilizing, mixing with appropriate non-toxic and non-interfering components, dividing into dose units, and enclosing in a delivery device.

(a) General modes of administration

Pharmaceutical compositions of the invention are administered by a mode appropriate for the form of composition. Typical routes include intravenous, subcutaneous, intramuscular, intraperitoneal, intradermal, oral, intranasal, intradermal, and intrapulmonary (i.e., by aerosol). Pharmaceutical compositions of this invention for human use are typically administered by a parenteral route, most typically intravenous, subcutaneous, intramuscular. Although not required, pharmaceutical compositions are preferably supplied in unit dosage form suitable for administration of a precise amount. Also contemplated by this invention are slow release or sustained release forms, whereby a relatively consistent level of the active compound are provided over an extended period.

(b) Liquid formulations

Liquid pharmaceutically acceptable compositions can, for example, be prepared by dissolving or dispersing a polypeptide or polynucleotide embodied herein in a liquid excipient, such as water, saline, aqueous dextrose, glycerol, or ethanol. The composition can optionally also contain other medicinal agents, pharmaceutical agents, carriers, and auxiliary substances such as wetting or emulsifying agents, and pH buffering agents. Compositions for injection can

be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to injection.

Pharmaceutical compositions for oral, intranasal, or topical administration can be supplied in solid, semi-solid or liquid forms, including tablets, capsules, powders, liquids, and suspensions. For administration via the respiratory tract, a preferred composition is one that provides a solid, powder, or liquid aerosol when used with an appropriate aerosolizer device.

The invention also encompasses compositions comprising liposomes with membrane bound peptide to specifically deliver the liposome to the area of the tumor or neoplastic cells or to the immune system. These liposomes can be produced such that they contain, in addition to peptide, immunotherapeutic agents such as those described above which would then be released at the site of malignancy. Wolff et al. (1984) *Biochem. Biophys. Acta* 802:259. Another such delivery system described by Brown et al. ((1994) *Virology* 198:477-488; and Miyamura et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8507-8511) utilizes chimeric parvovirus B19 capsids for presentation of the antigen-binding fragments. Such chimeric systems are encompassed for use in the claimed methods.

Compositions embodied in this invention can be assessed for their efficacy in a number of ways. Accordingly, test compounds are prepared as a suitable pharmaceutical composition and administered to test subjects. Initial studies are preferably done in small animals such as mice or rabbits, optionally next in non-human primates and then ultimately in humans. Immunogenicity is preferably tested in individuals without a previous antibody response. A test composition in an appropriate test dose is administered on an appropriate treatment schedule. It can be appropriate to compare different doses and schedules within the predicted range. The dosage ranges for the administration of anti-SPPC are those large enough to produce the desired effect in which the symptoms of the malignant disease are ameliorated without causing undue side effects such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication. Dosage can vary from about 0.1 mg/kg to about 2000 mg/kg, preferably about 0.1 mg/kg to about 500 mg/kg, in one or more dose administrations daily, for one or several days. Generally, when the compositions are administered conjugated with

therapeutic agents, lower dosages, comparable to those used for *in vivo* immunodiagnostic imaging, can be used.

2. Antigen-binding Fragments

The invention encompasses pharmaceutical compositions containing anti-SPPC. Such pharmaceutical compositions are useful for inducing or aiding an immune response and treating neoplastic diseases, either alone or in conjunction with other forms of therapy, such as chemotherapy, radiotherapy or immune therapies described in WO98/23735; WO98/34642; WO97/10000; WO97/10001; and WO97/06821.

Compositions containing antigen-binding fragments specific for disease-associated SPPCs and methods of use thereof, as described for cancer treatment, are also encompassed by the invention.

3. Immunogenic Compositions.

The SPPC compositions and SPPC peptide compositions of the invention can be used as cancer immunogens including vaccines. These compositions can comprise a cancer-specific antigen or epitope (e.g. one found on cancer cells but not on non-cancerous cells), which can be in the form of native peptides, artificial proteins, for example multiantigen peptides, branched polypeptides, fusion and recombinant peptides, as well as single T cell epitopes and tumor antigen peptides. Ben-Yedidia et al. (1997) *Curr. Opin. Biotechnol.* 8:442-8; and Hellstrom et al. (1997) *Mol. Med. Today* 3:286-90. A cancer vaccine can alternatively comprise a polynucleotide encoding an antigen, which is directly injected into muscle or skin to cause an immune response against the encoded antigen. Moelling (1997) *Cytokines Cell. Mol. Ther.* 3:127-35; and Moling (1997) *J. Mol. Med.* 75:242-6. Cancer vaccines can also comprise tumor cells. Mackensen et al. (1997) *J. Mol. Med.* 75:290-6. Anti-idiotype antigen-binding fragments are also suitable for use as vaccines.

A new method for generating useful tumor cell populations for such vaccines from tumor biopsies has been described. Lahn et al. (1997) *Eur. Surg. Res.* 29:292-302. Whole tumor cells used for this purpose can be lethally irradiated and transformed to produce a cytokine such as granulocyte-macrophage colony stimulating factor (GM-CSF). Mahvi et al. (1997) *Hum. Gene Ther.* 8:875-891; Stingl et al. (1997) *J. Mol. Med.* 75:297-9; and Jaffee et al. (1997) *Methods* 12:143-53. While both whole cells and cell lysates can be used as vaccines, whole cell vaccines

can induce a better immune response against cell-surface antigens. Ravindranath et al. (1997) *Anticancer Drugs* 8:217-24.

In a murine breast cancer model, Flt3-Ligand (Flt3-L), a stimulatory cytokine for a variety of hematopoietic lineages, including dendritic cells and B cells, has been used in conjunction with murine breast cancer cells as a vaccine. Chen et al. (1997) *Cancer Res.* 57:3511-6. Dendritic cells (DCs) can also be loaded with or transduced to express tumor antigens; these cells are then used as adjuvants to tumor vaccination. DCs present tumor-associated antigens endogenously to the afferent lymphatic system in the appropriate histocompatibility complex (MHC)-restricted context. Wan et al. (1997) *Hum. Gene Ther.* 8:1355-63; Peiper et al. (1997) *Surgery* 122:235-41; and Smith et al. (1997) *Int. Immunol.* 9:1085-93. Current melanoma vaccines manipulate antigen presentation networks and combine the best cellular and antibody antitumor immune response effective in mediating tumor protective immunity. These therapies have caused regression, delayed disease progression or an improvement in survival in some cases, with a paucity of side effects. Kuhn et al. (1997) *Dermatol. Surg.* 23:649-54. Melanoma vaccines are also reviewed in Conforti et al. (1997) *J. Surg. Oncol.* 66:55-64.

Vaccines can be packaged in pharmaceutically acceptable carriers or admixed with adjuvants or other components (such as cytokines) as is known in the art.

More specifically, an SPPC for use in a vaccine can comprise at least one polypeptide, which is an antigenic fragment, anti-idiotype of anti-SPPC, derivative, or variant of C-antigen or C-antigen peptide. Preferably the SPPC comprises an epitope of C-antigen. As used herein, the SPPCs are considered to be derived from a cellular membrane fraction of at least one cancer cell population. That is to say that the SPPC or epitope thereof can be found preferentially in the membrane fraction of disrupted and separated cells but the SPPC or portion thereof can be obtained in any manner including recombinant genetics. Thus, the SPPC or epitope thereof can be derived directly or indirectly from such a fraction. By "preferentially" in the membrane fraction, it is meant that more than 50%, preferably more than 75% and, even more preferably, more than 90% is found in the membrane fraction with corresponding amounts in the cytosolic or non-membrane fractions.

An epitope typically includes 5-10 amino acid residues. The C-antigen polypeptide comprises derivatives of C-antigen which preferably retain at least one epitope present on native,

whole C-antigen. This polypeptide can be administered as a vaccine in the form of free C-antigen polypeptide, C-antigen present on a cell expressing C-antigen; C-antigen in the context of multi-antigen peptides, branched polypeptides, fusion peptides, recombinant peptides; or C-antigens loaded onto dendritic cells (DCs). The cell expressing C-antigen can be a tumor cell naturally expressing C-antigen or a cell, which does not normally express C-antigen, which has been transformed with the C-antigen polynucleotide in order to express C-antigen. The cell can be irradiated or otherwise rendered non-viable. The C-antigen-expressing cell can also be altered (e.g. by transduction) to express a cytokine.

Vaccines for veterinarian use are substantially similar to that in humans with the exception that adjuvants containing bacteria and bacterial components such as Freund's complete or incomplete adjuvants, are allowed in the formulations.

4. Gene Therapy

The invention further encompasses methods of gene therapy and compositions for use therein. In one mode of gene therapy, the polynucleotides are used for genetically altering cells ex vivo. In this strategy, cells removed from a donor or obtained from a cell line are transfected or transduced with vectors encoding an anti-SPPC, and then administered to a recipient. Suitable cells for transfection include peripheral blood mononuclear cells.

In another mode of gene therapy, the polynucleotides of this invention are used for genetically altering cells in vivo. The purpose can include, but is not limited to, treating various types of cancer.

F. <u>Methods of Treatment</u>

Also included in this invention are methods for treating cancer. The methods comprise administering an amount of a pharmaceutical composition containing a composition of the invention in an amount effective to achieve the desired effect, be it palliation of an existing tumor mass or prevention of recurrence. For treatment of cancer, the amount of a pharmaceutical composition administered is an amount effective in producing the desired effect. An effective amount can be provided in one or a series of administrations. An effective amount can be provided in a bolus or by continuous perfusion. Suitable active agents include the anti-neoplastic drugs and bioresponse modifiers described above and effector cells such as those described by Douillard et al. (1986) *Hybridomas* (Supp. 1:5139).

Pharmaceutical compositions and treatment modalities of this invention are suitable for treating a patient by either directly or indirectly eliciting an immune response against neoplasia. An "individual", "patient" or "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to: humans, wild animals, feral animals, farm animals, sport animals, and pets. A "cancer subject" is a mammal, preferably a human, diagnosed as having a malignancy or neoplasia or at risk thereof.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the disease course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Therapeutic effects of treatment include without limitation, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastases, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

The "pathology" associated with a disease condition is any condition that compromises the well-being, normal physiology, or quality of life of the affected individual. This can involve, but is not limited to, destructive invasion of affected tissues into previously unaffected areas, growth at the expense of normal tissue function, irregular or suppressed biological activity, aggravation or suppression of an inflammatory or immunologic response, increased susceptibility to other pathogenic organisms or agents, and undesirable clinical symptoms such as pain, fever, nausea, fatigue, mood alterations, and such other disease-related features as can be determined by an attending physician.

An "effective amount" is an amount sufficient to effect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a patient in one or more doses. In terms of treatment, an effective amount is an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the disease, or otherwise reduce the pathological consequences of the disease. The effective amount is generally determined by the physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These factors include age, sex and weight of the patient, the condition being treated, the severity of the condition and the form and effective concentration of the antigen-binding fragment administered.

Suitable subjects include those who are suspected of being at risk of a pathological effect of any neoplasia, particularly carcinoma, are suitable for treatment with the pharmaceutical compositions of this invention. Those with a history of cancer are especially suitable.

Suitable human subjects for therapy further comprise two treatment groups, which can be distinguished by clinical criteria. Patients with "advanced disease" or "high tumor burden" are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, CAT scan, sonogram, mammogram or X-ray; positive biochemical or histopathologic markers on their own are insufficient to identify this population). A pharmaceutical composition embodied in this invention is administered to these patients to elicit an anti-tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

A second group of suitable subjects is known in the art as the "adjuvant group." These are individuals who have had a history of cancer, but have been responsive to another mode of therapy. The prior therapy can have included (but is not restricted to, surgical resection, radiotherapy, and traditional chemotherapy. As a result, these individuals have no clinically measurable tumor. However, they are suspected of being at risk for progression of the disease, either near the original tumor site, or by metastases.

"Adjuvant" as used herein has several meanings, all of which will be clear depending on the context in which the term is used. In the context of a pharmaceutical preparation, an adjuvant is a chemical or biological agent given in combination (whether simultaneously or otherwise) with, or recombinantly fused to, an antigen to enhance immunogenicity of the antigen. In the context of cancer diagnosis or treatment, adjuvant refers to a class of cancer patients with no clinically detectable tumor mass, but who are may be at risk of recurrence.

This group can be further subdivided into high-risk and low-risk individuals. The subdivision is made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different cancer. Features typical of high-risk subgroups are those in which the tumor has invaded neighboring tissues, or who show involvement of lymph nodes.

Another suitable group of subjects is those with a genetic predisposition to cancer but who have not yet evidenced clinical signs of cancer. For instance, women testing positive for a genetic mutation associated with breast cancer, but still of childbearing age, can wish to receive anti-SPPC treatment prophylactically to prevent the occurrence of cancer until it is suitable to perform preventive surgery.

A pharmaceutical composition embodied in this invention is administered to patients in the adjuvant group, or in either of these subgroups, in order to elicit an anti-cancer response. Ideally, the composition delays recurrence of the cancer, or even better, reduces the risk of recurrence (i.e., improves the cure rate). Such parameters can be determined in comparison with other patient populations and other modes of therapy.

Of course, crossovers between these two patient groups occur, and the pharmaceutical compositions of this invention can be administered at any time that is appropriate. For example, anti-SPPC therapy can be conducted before or during traditional therapy of a patient with high tumor burden, and continued after the tumor becomes clinically undetectable. Anti-SPPC therapy can be continued in a patient who initially fell in the adjuvant group, but is showing signs of recurrence. The physician has the discretion to determine how or when the compositions are to be used.

Various compounds and compositions of this invention have other clinical indications, of which the following section provides only a survey.

One indication is the treatment of cells ex vivo. This can be desirable for experimental purposes, or for treatment of an individual with a neoplastic disease. In one example, anti-SPPC is administered to a culture of cells, such as peripheral blood cells obtained from a donor, or a suitable cell line. About 0.5 to 2 μg/mL of anti-C can be an effective dose for this purpose. In a second example, donor cells are genetically altered with an expression vector of this invention, to provide for ongoing secretion of anti-SPPC after administration of the cells to the recipient.

Human cancer patients, including, but not limited to, glioblastoma, melanoma, neuroblastoma, adenocarcinoma, glioma, soft tissue sarcoma, and various carcinomas (including small cell lung cancer) are especially appropriate subjects. Suitable carcinomas further include any known in the field of oncology, including, but not limited to, astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal

adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bileduct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas.

The patients can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The patients can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.

"Immunologic activity" of an antigen-binding fragment refers to the ability to specifically bind the antigen which the intact antibody recognizes. Such binding can or can not elicit an immune response. A specific immune response can elicit antibody, B cell responses, T cell responses, any combination thereof, and effector functions resulting therefrom. Included, without limitation, are the antibody-mediated functions ADCC and complement-mediated cytolysis (CDC). The T cell response includes, without limitation, T helper cell function, cytotoxic T cell function, inflammation/inducer T cell function, and T cell mediated immune suppression. A compound (either alone or in combination with a carrier or adjuvant) able to elicit either directly or indirectly, a specific immune response according to any of these criteria is referred to as "immunogenic." Antigen-binding fragment "activity" or "function" refers to any

of the immunologic activities of an antibody, including the detection, amelioration or palliation of cancer.

An "immune response" refers to induction or enhancement of an immunologic response to malignant or diseased tissue, disease-causing agents and other foreign agents to which the body is exposed. Immune responses can be humoral, as evidenced by antibody production; and/or cell-mediated, as evidenced by cytolytic responses demonstrated by such cells as natural killer cells or cytotoxic T lymphocytes (CTLs) and the cytokines produced thereby. Immune responses can be monitored by a mononuclear cell infiltrate at the site of infection or malignancy. Typically, such monitoring is by histopathology. A "cancer-specific immune response" is one that occurs against the malignancy but not against non-cancerous cells. The treatments described herein typically induce or augment an antibody-mediated response but can also induce or augment a cell-mediated immune response.

When anti-SPPC is used in combination with various therapeutic agents, such as those described herein, the administration of both usually occurs substantially contemporaneously. The term "substantially contemporaneously" means that they are administered reasonably close together with respect to time. Usually, it is preferred to administer the therapeutic agent before anti-SPPC. For example, the therapeutic agent can be administered 1 to 6 days before anti-SPPC. The administration of the therapeutic agent can be daily, or at any other suitable interval, depending upon such factors, for example, as the nature of the malignancy, the condition of the patient and half-life of the agent.

Anti-SPPC enables therapies combining all of the characteristics described herein. For example, in a given situation it can be desirable to administer a therapeutic agent, or agents, prior to the administration of anti-SPPC in combination with effector cells and the same, or different, therapeutic agent or agents. For example, patients can be treated by first administering IFN-γ and IL-2 daily for 3 to 5 days, and on day 5 administering anti-SPPC in combination with effector cells, IFN-γ, and IL-2.

Therapeutic compositions can be administered by injection or gradual perfusion. Anti-SPPCs can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, intrathecally or transdermally, alone or in combination with effector cells.

Another method of administration is intralesionally, for instance by injection directly into the tumor. Intralesional administration of various forms of immunotherapy to cancer patients

does not cause the toxicity seen with systemic administration of immunologic agents. Fletcher et al. (1987) *Lymphokine Res.* 6:45; Rabinowich et al. (1987) *Cancer Res.* 47:173; Rosenberg et al. (1989) *Science* 233:1318; and Pizz et al. (1984) *J. Int. Cancer* 34:359.

Anti-SPPC is suitable for use in treating and imaging brain cancer. When the site of delivery is the brain, the therapeutic agent must be capable of being delivered to the brain. The blood-brain barrier limits the uptake of many therapeutic agents into the brain and spinal cord from the general circulation. Molecules that cross the blood-brain barrier use two main mechanisms: free diffusion; and facilitated transport. Because of the presence of the blood-brain barrier, attaining beneficial concentrations of a given therapeutic agent in the CNS can require the use of drug delivery strategies. Delivery of therapeutic agents to the CNS can be achieved by several methods.

One method relies on neurosurgical techniques. In the case of gravely ill patients, surgical intervention is warranted despite its attendant risks. For instance, therapeutic agents can be delivered by direct physical introduction into the CNS, such as intraventricular, intralesional, or intrathecal injection. Intraventricular injection can be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Omana reservoir. Methods of introduction can also be provided by rechargeable or biodegradable devices. Another approach is the disruption of the blood-brain barrier by substances which increase the permeability of the blood-brain barrier. Examples include intra-arterial infusion of poorly diffusible agents such as mannitol, pharmaceuticals which increase cerebrovascular permeability such as etoposide, or vasoactive agents such as leukotrienes. Neuwelt and Rappoport (1984) *Fed. Proc.* 43:214-219; Baba et al. (1991) *J. Cereb. Blood Flow Metab.* 11:638-643; and Gennuso et al. (1993) *Cancer Invest.* 11:638-643.

Further, it can be desirable to administer the compositions locally to the area in need of treatment; this can be achieved by, for example, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. A suitable such membrane is Gliadel® provided by Guilford sciences.

Another method involves pharmacological techniques such as modification or selection of the anti-SPPC to provide an analog which will cross the blood-brain barrier. Examples include increasing the hydrophobicity of the molecule, decreasing net charge or molecular

weight of the molecule, or modifying the molecule, such as to resemble one normally transported across the blood-brain barrier. Levin (1980) *J. Med. Chem.* 23:682-684; Pardridge (1991) in: *Peptide Drug Delivery to the Brain*; and Kostis et al. (1994) *J. Clin. Pharmacol.* 34:989-996.

Encapsulation of anti-SPPC in a hydrophobic environment such as liposomes is also effective in delivering drugs to the CNS. For example, WO 91/04014 describes a liposomal delivery system in which the drug is encapsulated within liposomes to which molecules have been added that are normally transported across the blood-brain barrier.

Yet another method takes advantage of physiological techniques such as conjugation of anti-SP-peptide to a transportable agent to yield a new chimeric, transportable, molecule. For example, vasoactive intestinal peptide analog (VIPa) exerts its vasoactive effects only after conjugation to a Mab to the specific carrier molecule transferrin receptor, which facilitates the uptake of the VIPa-Mab conjugate through the blood-brain barrier. Pardridge (1991); and Bickel et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:2618-2622. Several other specific transport systems have been identified, these include, but are not limited to, those for transferring insulin, or insulin-like growth factors I and II. Other suitable, non-specific carriers include, but are not limited to, pyridinium, fatty acids, inositol, cholesterol, and glucose derivatives.

G. <u>Additional Methods of Use</u>

1. Diagnostic Antibody Clearance

The invention also encompasses compositions and methods of use thereof in diagnostic antibody clearance. Anti-SPPC can be administered to an individual who has received a labeled anti-SPPC the course of radioscintigraphy or radiotherapy to remove the label. Effective imaging using radiolabeled antibodies is hampered due to excess circulating radiolabeled antibody, which often takes several days to clear. Accordingly, the SPPC recognized by the anti-SPPC is administered to the individual at a specified time after administration of the labeled anti-SPPC. Antigen that is complexed with the antigen-binding fragments at sites other than the tumor, such as in the circulation and interstitial spaces, promotes clearance of non-bound antibody and decreases background radiation. As a result, the level of label in unaffected tissues is reduced, and the image of the tumor (in comparison to neighboring tissues) is enhanced.

Similarly, when radionucleotides are given to subjects for irradiation of a tumor site, it is desirable to reduce collateral exposure of unaffected tissue. This invention thus includes

methods of treatment in which a radiolabeled anti-SPPC is administered in a therapeutic dose, and followed by administration of a molar excess of SPPC.

2. Imaging/Diagnostic, in vitro

The invention further encompasses methods for *in vivo* detection of antigen. A diagnostically effective amount of detectably labeled anti-SPPC is given to the subject in need of tumor imaging. The term "diagnostically effective" means that the amount of detectably labeled anti-SPPC is administered in sufficient quantity to enable detection of the neoplasia.

The concentration of detectably labeled anti-SPPC which is administered should be sufficient such that the binding to those cells having tumor-associated SPPC is detectable compared to the background. Further, it is desirable that the non-bound labeled antigen-binding fragment be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled antigen-binding fragment for *in vivo* diagnosis is somewhat patient-specific and depends on such factors as age, sex, and extent of disease. The dosage can vary from about 0.01 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages can vary, for example, depending on number of injections given, tumor burden, and other factors known to those of skill in the art.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay, which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the individual is minimized. Ideally, a radioisotope used for *in vivo* imaging lacks a particle emission, but produces a large number of photons in the 140–250 keV range, to be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes can be bound to anti-SP-peptide either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylene triaminepentacetic acid (DTPA) and

CA 02290722 1999-12-08

ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, ^{90}Y, ^{99m}Tc and ^{201}Tl.

Antigen-binding fragments can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually, gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}DY, ^{52}Cr, and ^{56}Fe.

3. Imaging/diagnostic, in vitro

Antigen-binding fragments can also be used to detect neoplasias using *in vitro* assays. Biological samples are taken from the patient and subject to any suitable immunoassay with anti-SPPC to detect the presence of tumor-associated SPPCs. This is particularly useful in detecting lymphomas and leukemias where the tumor cells are circulating in the patient's bloodstream.

A "biological sample" encompasses a variety of sample types, including blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimens or tissue cultures, or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term encompasses various kinds of clinical samples obtained from any species, and also includes cells in culture, cell supernatants, cell lysates and fractions thereof. Particularly, for the purposes described herein, biological samples comprise tumor tissue or tissue thought to be tumorous and are obtained for instance by surgical resection, biopsy, aspiration or any method known in the art.

4. Therapeutic Monitoring.

Antigen-binding fragments can also be used to monitor the course of amelioration of malignancy in an individual. Thus, by measuring the increase or decrease in the number of cells expressing tumor-associated SPPC or changes in the concentration of the complex present in various biological samples, it is possible to determine whether a particular therapeutic regimen aimed at ameliorating the malignancy is effective.

Phage Display Library Recognizing Consensus Conforming Peptides

It is to be understood that potentially suitable genetic packages include cells, spores and viruses (see US Patent No. 5,571,698), namely replicable genetic packages. Preferably, the replicable genetic package is a recombinant phage and said heterogeneous population of replicable genetic packages collectively constitute a phage display library.

Interpretation

Unless otherwise implied or stated, the term "biasing" and related forms of this term, are generally intended to refer to weighting in the course of introducing variation in the parental binding-fragment.

It is to be understood, for example, that 90% biasing in favor of wild-type amino acids at a given amino acid position is to be approximated by controlling the percentage amounts of each of the three relevant nucleotides (so that, for example, the product of the probabilities of occurrence of the three desired nucleotides in sequence in the growing chain is 90%) so as to supply 90% of correct coding triplet(s) and a total of 10% of random coding triplets, having regard to the degeneracy of the genetic code (for example if two different coding triplets result in a given amino acid, then the sum of the probabilities of achieving those two triplets will have to equal 90%). This is preferably accomplished on an amino acid by amino acid basis so that, for example the probability of achieving two and three wild-type amino acids in sequence, in the case of 90% biasing is 0.81 and 0.73, respectively, etc.

It is to be understood that this high level of biasing can be suitable only for part of the coding sequence into which variability is introduced and that higher levels of biasing are acceptable, when for example substantially all of the amino acids of a long CDR3 are biased, as disclosed in one of embodiments herein. Accordingly there is a balance to be struck between a large diverse library and biasing for maintaining parental binding fragment characteristics. Nevertheless it is contemplated, in another aspect of invention that the final library can be a pooled library in which several libraries each having varying degrees of biasing to wild-type, for example, 60%, 50%, 40% and 30%, are pooled together to obtain the both desired variability and similarity.

It is to be understood that biasing of a percentage less than 100% implies unless otherwise implied or stated that the remaining percentage is fully randomized.

Unless otherwise specified, a given "% biasing" or "% of binding-fragments" (or "biasing 10-100%", etc.) refers to biasing on an individual amino acid basis (though other techniques to accomplish the same effect might apparent to those skilled in the art).

Similarly, the specification that wild-type amino acids occur at a specified position or series of positions in, for example, at least approximately 50% of potential binding-fragments is intended to mean both that 50% biasing is sought at a given such position or that a total of 50% of the correct nucleotide triplets are represented. The use of the term "approximately" in reference to percentages is intended to accommodate attrition of various desired potential binding-fragments, the inaccuracy of the assumption that the probabilistic outcomes will be achieved in practice and that certain variation in methods to accomplish the specified results is deemed to be suitable.

The practice of the invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Wei & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991). These references are incorporated herein by reference. These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, can be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

Recombinant genetic techniques have allowed cloning and expression of antibodies, functional fragments thereof and the antigens recognized. These engineered antibodies provide novel methods of production and treatment modalities. For instance, functional immunoglobulin fragments have been expressed in bacteria and transgenic tobacco seeds and plants. Skerra (1993) *Curr.Opin. Immunol.* 5:256:262; Fiedler and Conrad (1995) *Bio/Technology* 13:1090-1093; Zhang et al. (1993) *Cancer Res.* 55:3384-3591; Ma et al. (1995) *Science* 268:916; and, for

a review of synthetic antibodies, see Barbas (1995) *Nature Med.* 1:836-839. These and more current references describing these techniques, which these references, particularly those well known to persons practicing in the relevant arts, are hereby incorporated herein by reference.

Nucleotide sequences can be isolated, amplified, and processed by standard recombinant techniques. Standard techniques in the art include digestion with restriction endonucleases, and amplification by PCR, or a suitable combination thereof. PCR technology is described in U.S. Patent Nos. 4,683,195; 4,800,159; 4,754,065; and 4,683,202, as well as *PCR: The Polymerase Chain Reaction*, Mullis et al., eds., Birkauswer Press, Boston (1994).

Polynucleotides comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides can be introduced into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, f-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell by standard methods. See, e.g., Sambrook et al. (1989). RNA can also be obtained from transformed host cell, or it can be obtained directly from the DNA by using a DNA-dependent RNA polymerase.

Suitable cloning and expression vectors include any known in the art, e.g., those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors and suitable host cells are known in the art and are not described in detail herein. See e.g. Gacesa and Ramji, *Vectors*, John Wiley & Sons (1994).

Phage display techniques are generally described or referenced in some of the preceding general references, as well as in U.S. Patent Nos. 4,593,002; 5,403,484; 5,837,500; 5,571,698; 5,750,373; 5,821,047; 5,223,409 and 5,702,892. "Phage Display of Peptides and Proteins", (Kay, Brian K. et al., 1996); "Methods in Enzymology", Vol. 267 (Abelson, John N., 1996); "Immunology Methods Manual", (Lefkovits, Ivan, 1997); "Antibody phage display technology and its applications", (Hoogenboom, Hennie R. et al., 1998). Immunotechnology 4 p.1-20.

Generally, DNA encoding millions of variants of a parental binding-fragment can be batch-cloned into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI or pVIII). Upon expression, the coat protein fusion will be incorporated into new phage particles that are assembled in the bacterium. Expression of the fusion product and

its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. This connection between ligand genotype and phenotype allows the enrichment of specific phage, e.g. using selection on immobilized target. Phage that display a relevant ligand will be retained, while non-adherent phage will be washed away. Bound phage can be recovered from the surface, reinfected into bacteria and re-grown for further enrichment, and eventually for analysis of binding. The success of ligand phage display hinges on the combination of this display and enrichment method, with the synthesis of large combinatorial repertoires on phage. Creation of single chain Fv's and other important methods are generally described in U.S. Patent Nos. 4,946,778; 5,260,203; 5,482,858; 5,258,498 and 5,525,491.

PPEs

SPPCs, wherein the peptide portion is a consensus conforming peptide, can be used to create effective treatment against tumor in the manner described, for example, in use Patent Nos. 5,750,119; 5,830,464; 5,837,251; 5,948,646; 5,935,576; WO 97/06685 and WO 99/22761, the disclosures of which are hereby incorporated by reference. In one embodiment it is contemplated that a composition enriched for consensus conforming peptides as presented by PPEs. Preferably the composition at least predominately comprising such PPEs.

PPEs present the consensus conforming peptides in a manner in which they share antigenic determinants with SPs e.g. HSP70s and HSP90 so that for a given peptide the PPE will be immunogenic with respect to that peptide as presented by a SP. It follows that PPEs are preferably SP - CCP complexes wherein, the SP is preferably the same SP as the SPPC targeted. PPEs include APCs and other well known peptide presenting scaffolds (see e.g. Hoogenboom 1998 referenced above, which discusses and refers to several of these). It is contemplated that the non-CCP portion of the PPE can be a substrate presents different CCPs on different locations on the substrate preferably within a grid in which each individual CCP is preferably characterized by its specific amino acid sequence and location on the grid. Alternatively, with respects to aspects of the invention calling for a library of CCPs, individual CCPs can synthesized, reconstituted with commercially available SPs (e.g. sourced from Stressgen) and plated out in wells in a manner conducive for high through put screening. For example, peptide microarrays for antibody screening can be accomplished in the following manner: peptides candidate conforming to the consensus sequence for MAb H11 and SP binding are expressed as

bacteriophage fusion proteins, other fusion proteins or synthetically synthesized and tethered to the surface of microchips, polymer beads or conventional plastic ware (e.g. 96-well plate) or similar display systems. A human combinatorial antibody (antibody fragment) phage display library is generated and used to screen against the array of consensus conforming peptides. Positive binding antibodies and their corresponding peptide antigens are identified by the sequence of the peptide to which they bind. For recombinant peptide protein fusions, control antibody screening is performed against the bacteriophage without peptide. Positive binding is considered to be two times the signal to noise ratio above the control. Screening of tumor cells or tumor cell surface extracts (fresh, frozen or fixed or other method of preparing tumor cells or tumor cell surface extracts), either individually or in a display system described above. Correlation of the cancer type with the peptide type is used to establish the relationship between individual peptides and cancer types. Ultimately, the cancer specific peptide(s) could be used as candidate immunogens to induce an anti-tumor response specific for the specific tumor type. Various methods for high through put screening are described in the art. Reference is made to Specer, R.W. *Biotechnol Bioeng* 1998 Winter 61:1 61-7; McDonald, O.B. et al. *Anal Biochem* 1999 Mar 15 268:2 318-29; Crameri, R. et al. *Comb Chem High Throughput Screen* 1999 Apr 2:2 63-72; Platonova, G.A. et al. *J. Chromatogr A* 1999 Aug 6 852(1) 129-40; Crameri, R. et al. *Comb Chem High Throughput Screen* 1999 Apr 2(2) 63-72; Lueking, A. et al. *Anal Biochem* 1999 Can 15 270(1) 103-11; Schullek, J.R. et al. *Anal Biochem* 1997 Mar 1 246:1 20-9; Kay, B.K. et al. *Mol Divers* 1996 Feb 1:2 139-40.

Alternatively, individual CCP-SP complexes can be generated by creating, via PCR, a variety of polypeptides that have a repeating consensus motif e.g.[$(HyX\ HyX\ HyX\ Hy)_n$ wherein $n = 2$ to 50] and individually translating into separate populations of cells so that they are naturally associated with SPs after proteolysis, within the cells, and later extracted for use as a source of SP complexes according well known method (see for example U.S. Patent Nos. 5,750,119; 5,830,464; 5,837,251; 5,948,646 and 5,435,576 and references disclosed therein).

For "cellular loading" of endogenous SPs with exogenous-derived CCPs: one optional host cell line is one that down-regulates MHC expression, i.e., most human cells. In one embodiment, where the exogenous CCP is not expressed in sufficient abundance to be identifiable by its preponderance (e.g. evaluating the degree of binding to a given antigen-binding fragment specific for HS-CCP complexes) strategies can be employed to decrease

competition for SPs by host-derived proteins/peptides the cells are infected by a virus for example, which takes over the host cell's protein synthesis machinery such that almost all of the protein produced by the cell is viral proteins. One can modify the mammalian virus outer coat structural protein by introducing a CCP sequence in a location of the viral protein that does not adversely affect virus assembly and cell infection. Individual CCP sequence virus libraries can be produced in this manner. To create a library one can transfect each individual wells/plates/flasks containing the appropriate host cell line (e.g. Daudi) with a specific recombinant-CCP-containing virus and incubate for an appropriate period and then isolate SPPCs from each specific infection and analyze for CCP content.

According to another embodiment of the invention nucleic acids encoding CCPs can be used for immunization in the form of a "DNA vaccine", according to well-known methods.

SP-CCP Complexes: Other Methods of Production

In another embodiment of the invention heat shock proteins bound to consensus conforming peptides (CCPs) can be generated intracellularly by synthetically generating a polynucleotide encoding a plurality of such CCPs as segments within larger peptide. Optionally, enzymatic peptide cleavage sites can be introduced between the various CCP segments to ensure intracellular cleavage of intact segments. These polynucleotides are optionally amplified prior to introduction into a host cell for expression. The polynucleotides are then inserted into an expression vector or intrachromosomally integrated, operatively linked to regulatory element(s) such as a promoter, for purposes of expressing the encoded proteins in suitable host cells in vitro. Reference is made to US Patent No. 5,948,646, which describes analogous methods using cancer cell DNA, the disclosure of which is hereby incorporated by reference.

The polynucleotides are introduced into host cells where they are expressed by the host cells, thereby producing intracellularly noncovalent complexes of SPs and peptides (including those peptides encoded by the polynucleotides). The recombinant host cells can be cultured on a large scale for production of large amounts of the immunogenic complexes. The polynucleotide library can be stored for future use (e.g. by lyophilization or freezing), or expanded by replication in a cloning vector in suitable host cells to meet increased demand for the subject immunogenic complexes.

Optionally, the host cell is a cancer cell, optionally which expresses SPPCs on its surface, optionally the same type of cancer as the tumor target, optionally cancer cells of individual sought to be treated by the SP-CCP complexes.

Methods of Purifying SPPCs

A variety of methods have been proposed in the literature for purifying SPPCs. Reference is made to WO95/24923 and to more recent. US Patent No. 5,948,646 (Srivastava et al.) and WO99/29182, the disclosures of which are hereby incorporated by reference.

Consensus Sequences

According to one embodiment of the invention, the consensus sequence is HyXHyXHyXH, as described in Blond-Elguindi et al., 19 Nov. 1993, Vol. 75 pages 717-728. Consensus conforming peptides are preferably those that are represented within nature. According to another embodiment of the invention, with respect to human tumors, consensus conforming peptides that are represented within human cells are preferred, inasmuch as these proteins are the potential source of consensus conforming peptide that are picked up by SPs and brought to the surface of the cell.

According to another embodiment of the invention, the consensus sequences were generated by panning against H11 antibody described in published PCT Application No. PCT/US97/08962, an antibody that recognizes heat-shock protein peptide complexes on a number of different tumors, and to SP peptide complexes before treatment with ATP but not after. Other preferred consensus peptide motifs are described throughout the application.

Example 1

Suitable phage display libraries including, but not limited to the Ph.D. Phage Display 12-mer peptide library (NEB) were panned against the H11 antibody (including and not limited to the IgM, IgGI, scFv and other antibody fragments) exactly as described in the relevant NEB technical bulletin. See page 11 Sloan Kettering Patent WO 99/22761 for full details. Phage particles were prepared from individual clones and DNA was extracted and sequenced using the Applied Biosystems automatic sequencer and the deduced amino sequences were obtained. Following three rounds of panning the library was enriched for the following sequences:

7-mer peptide library

2. Consensus: [H] [+] [+] [H] [U] [H/-] [H/U]

CA 02290722 1999-12-08

	6	6		6			6		6		5/1
4/2	H		R		Y		S	L	P		
	F	H		R		Y		S	D	Y	
	F	H		R		Y		S	P	T	
	F	H		R		Y		T	P	G	
	F	H		R		Y		S	L	P	
	M	H		R		Y		T	P	L	

12-mer peptide library

Consensus:		[H]	[+]	[H/U]	[U/H/+]		[H/U]	[H/U/-]	[U/H]	
	8	8	6/2	4/3/1			6/2	5/2/1		4/4
	V	R	L	Q		P	G	A		
	M	H	P	W		P	T	Q		
	I	H	W	S		L	V	P		
	W	H	W	S		W	I	Q		
	F	H	W	P		T	L	Y		
	M	H	G	L		N	A	N		
	V	R	G	H		L	D	P		
	W	H	W	T		W	P	N		

Where (H) = Hydrophobic amino acid

(+) = Positively charged amino acid

(U) = Uncharged amino acid

(-) = Negatively charged amino acid

3. SEQUENCES : FHRYSLP

FHRYSDY
FHRYSPT
FHRYTPG
FHRYSLP
MHRYTPL
YHVRLQPGAAAA
AQSMHPWPTQSL
IHWSLVPWSNRS
WHWSWIQNAAPN
FHWPTLYNMYIP
QLQMHGLNANRQ
VRGHLDPPEAWP
WHWTWPNMTIPQ

Amino acids were grouped according to Molec. Cell Biol., 2 ed. 1990; Darnell et al. W.H. Freeman and Co.

7-mer Peptide library analysis

Residue 1: INVARIABLE – Hydrophobic [F_5M]

Residue 2: INVARIABLE – Basic [H_6]
Residue 3: INVARIABLE - Basic [R_6]
Residue 4: INVARIABLE - Hydrophobic [Y_6]

A. Residue 5: VARIABLE – Uncharged [S_4T_2]

B. Residue 6: VARIABLE - Predominantly hydrophobic [P_3L_2/D]

C. Residue 7: VARIABLE – Hydrophobic/ Uncharged [P_2LYTG]

12-mer peptide library analysis

Residue 1: INVARIABLE – Hydrophobic [$V_2M_2IW_2F$]
Residue 2: INVARIABLE – Basic [H_6R_2]
Residue 3: VARIABLE - Predominantly Hydrophobic [W_4LP/G_2]
Residue 4: VARIABLE - Predom. Uncharged/Hydrophobic [S_2TQ/WP/H]

D. Residue 5: VARIABLE – Predom. Hydrophobic/Uncharged [$P_2L_2W_2$/TN]

E. Residue 6: VARIABLE - Predominantly Hydrophobic [VILAP/GT/D]

F. Residue 7: VARIABLE – Predom. Hydrophobic or Uncharged [Q_2YN_2/AP_2]

Phage isolated from the panning were also panned against the Hsc 70 and bound the same peptides. J. Biol. Chem. Vol 270; 19839 (1995). Panning a 15-mer peptide phage display library with Hsc70 resulted in the following observations:

Binding peptides require:

1. Peptide contains large hydrophobic internal residues.
2. Enrichment for basic residues.
3. Substitution of basic residues results in reduction of binding affinity.
4. Comparison of peptide binding to Hsc 70, BiP and DnaK indicated that some residues were common whilst others were exclusive and binding can be fine-tuned.

See also Proc. Natl. Acad. Sci USA 87:6378 (1990); and Cell Vol. 75:717 (1993).

Panned peptide 8-mer and 20-mer peptide display library; BiP 70 preferentially binds:

1. Peptides containing aromatic and hydrophobic amino acids in alternating positions.
2. Peptides bind in extended conformation with side chains of alternating amino acids pointing into the cleft of the BiP 70 molecule.

3. Synthetic peptides could be reassociated with BiP in vitro and shown to stimulate its ATPase activity.

Example 2

Peptide-SP Reconstitution protocols

SP 90 or SP 70 and associated peptides are mixed in appropriate concentrations For example (but not limited to) 1 ug of peptide to 9 ug of SP or 1 molar ratio of 5 peptide molecules to 1 molecule of SP.

The mixture is incubated for 2 h at 30^0C with an incubation buffer consisting of 3 mM $MgCl_2$, 1 mM PMSF, at pH 7.2. Excess or unbound peptides are removed by centrifugation through a Centricon 10 concentrator. Alternatively, SP 90 and associated peptides can be incubated in Na_2PO_4 buffer at 50^0C for 10 min followed by incubation at room temperature for 30 minutes.

HSP70 and associated peptides are incubated at 37^0C in sodium phosphate buffer containing 1 mM ADP and 1 mM $MgCl_2$. Free peptides are removed as above. (Reference, Blachere, N.E et al; J. Exp. Med. 186, 1315 (1997) for details).

Example 3

Introducing genetic variation into the sequence corresponding to the H11 heavy chain CDR3 region: Oligonucleotides comprising randomly mutated CDR3 regions were prepared on an Applied Biosystems 394 DNA synthesizer as described above.

This anti-codon formula [(A/C)NN], is used and results in a reduction in possible codon usage from 64 to 32 and reduces the number of possible stop codons. Position one, therefore, comprises only A and C in the synthetic reaction mixture. For complete randomization of the second and third positions of the codons the dNTP mixture comprises 25% A,G,C and T.

The 3' oligonucleotide randomizing primer was designed such that the last 15 nucleotides of framework 3 and the first 16 nucleotides of framework 4 were kept constant for hybridization. The nucleotides encoding the intervening amino acids, namely all the amino acids of the CDR3 region except 101 and 102 (Kabat numbering system, Kabat et al. 1991) were randomized.

Selective randomization for phage display of consensus binding fragments: Methods of generating scFv and binding fragment libraries are well known in the art.

For maintaining various percentages of wild type amino acid residues: This is achieved by creating residue substitutions by using different spiking levels of the various dNTPs as described below.

To achieve approximately 50% biasing to wild type at any one position in the H11 CDR3 region during antisense synthesis using the DNA synthesizer , the following example would be used. In the case of tyrosine, which is encoded by TAC or TAT (antisense strand GTA or ATA) the nucleotides would be spiked as follows for the antisense strand.

The nucleotide spiking levels would be as follows:

First anticodon nucleotide position: Only 80% of A and 20% of C is added, G and T are not added to reduce codon degeneracy.

Example 4

Compositions of CCPs

Suitable compositions of CCPs for use in a conservative approach (starting with a smaller number of CCPs to determine their representation on tumor cells, as opposed to using some of the larger subset disclosed herein) to screening CCPs for tumor-relatedness include evaluation of the following two subsets of consensus conforming peptides generated from 7 mer and 12 mer peptide library panning of H11 binding sit, respectively, are as follows:

This was generated on Genbank with the 7 amino acid consensus sequence [PAILMFWVY][KRH][KRH][PAILMFWVY][STNQG][PAILMFWVYED][PAILMFWVYSTNQG], which corresponds to the consensus sequence of H + + H U H/- H/U

CA 02290722 1999-12-08

Consensus Conforming Sequence:

PHHPGFL	YKKWQEN	FKHVSPA	AKKLQAM	LKHVNAL
LKKYGFS	WKRYNIL	VKRVSEN	AKKLQAM	MKRLSYI
VHRPGFY	AKHVQFV	ARKIQVP	AKKLQAM	PKRWSVI
VHRPGFY	AKHVQFV	ARKIQVP	MKRLGMF	PKRWSVI
VHRPGFY	IKHPNLV	FKKAQEL	LHHLTIP	PKRWSVI
VHRPGFY	IKHPNLV	FKKISEG	ARRLTLA	PKRWSVI
VHRPGFY	AHRLGLF	FKKISEG	ARRLTLA	PKRWSVI
VHRPGFY	YKRVGAL	FKKISEG	VHRLSML	AKKFGVL
VHRPGFY	YKRVGAL	FKKISEG	IKKVSYN	PRKAGVF
AKKMGLV	YKRVGAL	FKKISEG	IKKVSYN	PRKAGVF
MHKAQLV	YKRVGAL	FKKAQEL	LKKLSFY	FHHPTYG
AHKIQVQ	AKRFQAQ	AKHLTPV	IKKVSYN	FHHPTYG
AKKLSMY	AKRFQAQ	AKHLTPV	IKKVSYN	IRRYSDA
AKRLGYI	AKRFQAQ	AKHLTPV	VRKANDI	IRHFTDS
FKRVGLA	AKRFQAQ	AKHLTPV	VRKANDI	IRRYSDA
AHHLGPA	AKRFQAQ	AKHLTPV	LRHISPQ	YRRLQYL
AHHLGPA	AKRFQAQ	LRRISLS	LRHISPQ	YRRLQYL
LKRLQPG	PRRVGIA	AKKLQPI	LRHISPQ	YRRLQYL
FHRPSEL	PRRVGIA	AKKLQPI	LRHISPQ	YRRLQYL
FHRPSEL	VKKVNES	AKKLQPA	IKKANEV	PKRISPV
LRRLQDV	AKRPGIY	AKKLQPA	IKKANEV	PKRWSLI
LRRLQDV	ARRLTVS	AKKLQPA	IKKANEV	AKKPTAA
LRRLQDV	LRKPGLP	AKKLQPA	IKKANEV	IKKVQDL
LRRLQAV	LKHPGLA	IHHWNAN	LRHISPQ	IKKVQDL
VKKVNAV	YHRINEG	LHHLTIP	LRHISPQ	IKKVQDL
MKRLGMF	YHRINEG	FRRITLY	LRHISPQ	IKKVQDL
VKKFNDW	WKKVQLQ	MRRISLF	LRHISPQ	AKRLGYI
PKKMTDY	ARKIGVV	MKRLGMF	LHRLQVS	AKRLGYI
LRRLQAQ	VRHMQEW	LHKIGAL	VRRAQPP	AKRLGYI
VRKFQIP	FKRLNIN	LHKIGAL	VRRAQPP	AKRLGYI
ARRPTPN	FKRLNIN	LKKLSFY	VRRPSLQ	VKKIQES
VKHVTVI	FKRLNIN	LKKLSFY	VRRPSLQ	VKKIQES
IKKISPF	FKRLNIN	AKKASAF	VRRPSLQ	VKKIQES
IKKISPF	FKRLNIN	AKKASAF	VRRPSLQ	VKKIQES
IKKISPF	AHHASEN	LRRANLT	VRRPSLQ	LRHYSWA
LKKATAY	AHHASEN	LRRANLT	VRRPSLQ	LRHYSWA
FRRLSDQ	AHHASEN	IRRMNEN	VRRPSLQ	VKKPGAS
FRRLSDQ	AHHASEN	IRRMNEN	VRRPSLQ	VKKPGAS
FRRLSDQ	LRKIQES	MKRLGMF	VRRPSLQ	VKKPGAS
FKHVSPA	LRHFSVT	FKKPTPS	VRRPSLQ	VKKPGES
AHRPTVG	VRHITEQ	WKKLQLG	MKKVTIS	VKKPGES
VRHATVT	PHHPGFL	IHRLSLM	WKHLSDI	LKKPGES
VKKLGFN	PHHPGFL	MKRLGMF	WKHLSDI	VKKPGES
VKKLGFN	PHHPGFL	MKRLGMF	AKKASAF	LKKPGES
PHHYSEP	LRRLQLL	MKRLGMF	AKKASAF	LKKPGES
YHKPQFA	LRRLQLL	LHHLTIP	AKKASAF	LKKPGES
YHKPQFA	AKHMGVM	LHHLTIP	LRRATIS	LKKPGES
YHKPQFA	AKKLQPI	AKKLQAM	IRKLSDQ	LKKPGES
AKRFQIN	FRKLQDA	AKKLQAM	AKRLGYI	LKKPGES
LRKLGLG	LRRLNAT	AKKLQAM	PRHISVA	VKKPGES
MKHINLS	VHKLSYY	AKKLQAM	PHKINPL	LKKPGES
PRRLSLG	MKHMQVN	AKKLQAM	PHKVSAG	VKKPGES
WHKLGIS	LHHPSEA	AKKLQAM	PHKVSAG	MKKPGES
PRRLSLG	LHHPSEA	AKKLQAM	FKKISEG	MKKPGES

	MKKPGES		LHKFNEF		YRRFTEI		IKHVGPS	225	LRHPTAL
	MKKPGES		LHKFNEF		LRRLSAG	170	PHRINIY		FKRLGEL
	MKKPGES		LHKFNEF	115	FKKATVT		PHRINIY		AKKITAA
	MKKPGES	60	LHKFNEF		FKKATVT		PKKAGDI		AKKLQAM
5	MKKPGES		LHKFNEF		IHKLQDW		PKKAGDI		AKKLQAM
	MKKPGES		LHKFNEF		IHKLQDW		AKKVNWI	230	LRRIQPP
	MKKPGES		LHKFNEF		IHKLQDW	175	AKKVNWI		LRRIQPP
	MKKPGES		LHKFNEF	120	VKRAGLN		VHRISIF		LRRIQPP
	MKKPGES	65	LHKFNEF		VKKVQAF		IHKLQDW		LRRIQPP
10	MKKPGES		LHKFNEF		VKKINES		IHKLQDW		FRHFNPG
	LKKPGES		LHKFNEF		PRRAGPI		IHKLQDW	235	LRRIQPP
	VKKPGES		LHKFNEF		PRKPSAP	180	IHKLQDW		YRKLSMQ
	VKKPGES		LRKYTYN	125	ARRPGAA		IHKLQDW		YRKLSMQ
	VKKPGES	70	VKKMTVT		ARRPGAA		IHKLQDW		IKRWQAI
15	LKKPGAS		VKKMTVT		ARRPGAA		LRRMSVI		LRHLNFT
	VKKPGES		VKKMTVT		ARRPGAA		AKRANAN	240	LRHLNFT
	PKKPSAG		VKKATVV		ARRPGAA	185	AKRANAN		AKKLGLT
	AKRLQVN		VKKATVV	130	PRHISVA		AKRANAN		VKRIQEN
	AKRLQVN	75	PHRAQPA		VKKATVV		AKRANAN		VKKINES
20	AKRLQVN		PHRAQPA		LKKPSET		ARRLGWL		FKKANMA
	FRKLTPS		PKRATEM		LKKPTET		ARRLGWL	245	MRKLQLG
	FRKLTPS		VRKWSLN		FRHWNEW	190	AKRANAN		MRKLQLG
	FRKLTPS		VKKWGIN	135	YRHWNEW		AKKYTEF		WKRPTEL
	FRKLTPS	80	IKHPNLV		FKRISEQ		FRKPSYI		VRHLGIV
25	FKHLGFT		IKHPNLV		PRHISVA		FRKPSYI		FRKLNFN
	VKKPGES		LRKAGLL		PRHISVA		FKKFNEI	250	FRKLNFN
	VRKPGAS		LRKAGLL		PRHISVA	195	FKKFNEI		FRKLNFN
	VKKPGAS		IRKINEL	140	PRHISVA		FKKFNEI		FRKLNFN
	VKKPGAS	85	VKHLQVF		PRHISVA		FKKFNEI		FRKLNFN
30	IKHLSVN		VKHLQVF		PRHISVA		FKKFNEI		ARKLSEQ
	IKHLSVN		VKHLQVF		IRHLGWL		FKKFNEI	255	ARKLSEQ
	FKRIGYL		VKHLQVF		LKKVTMN	200	FKKFNEI		AHRLQFW
	PHHYTPI		YRHWQIP	145	LKKVTMN		ARRPGAA		AHRLQFW
	LHKFNEF	90	YRHWQIP		LKKVTMN		VHKLSYY		LRRANPS
35	LHKFNEF		LHHPSEA		VKKATVV		VHKLSYY		LRRANPS
	LHKFNEF		IRKVQFA		VKKATVV		VHKLSYY	260	FKRMTAL
	LHKFNEF		VRKVQFA		IKKPSLV	205	YRKITIN		VRRIQPL
	LHKFNEF		VRKVQFA	150	IRKVQYA		WKRASIQ		ARHPQLL
	LRKMGAP	95	VRRPQAV		IRKVQYA		LKKLGVN		LRHLNFT
40	LRKMGAP		PRKVNLG		IRKVQYA		LKKLQVN		LRHWGWG
	LKKLTIS		IHHFNEG		IRKVQYA		VHKLSYY	265	LRHWGWG
	LKKLTIS		IHHFNEG		FRHWNEW	210	VKKYQEG		LRHWGWG
	LHKFNEF		MKRYSMY	155	LHRLQVS		VKKYQEG		ARHPSFF
	LHKFNEF	100	FHKPSYF		IHKYNAY		LHKVNFY		IRHPQYN
45	LHKFNEF		VKRLTDA		IHKYNAY		PRRVGIF		ARHPSFF
	LHKFNEF		VKRLTDA		LKKPTET		FRHLGES	270	ARHPSFF
	LHKFNEF		VKRLTDA		FKRISEQ	215	YKKFGAT		ARHPSFF
	LHKFNEF		VKRLTDA	160	FKRISEQ		LKRLSLG		LRKFGVP
	LHKFNEF	105	LRRLTPS		FKRISEQ		LKRLSLG		LRKFGVP
50	LHKFNEF		LRRLTPS		FKRISEQ		LKHAQDS		LRKFGVP
	LHKFNEF		FHHLTVA		LKRVSEQ		MKHLTVQ	275	LRKFGVP
	LHKFNEF		FHHLTVA		LKRVSEQ	220	MKHLTVQ		VRRLTVG
	LHKFNEF		AHRLNIA	165	PHRFNDT		MKHLTVQ		LHKVTYL
	LHKFNEF	110	ARRLSLM		FKKVSLL		LRHPQPG		LKKLGIL
55	LHKFNEF		AKKFSAV		LKRATVQ		LRHPQPG		LKKLGIL
	LHKFNEF		AKKFSAV		IKHVGPS		LRHPTAL	280	LKKLGIL

AKRLQVN	AKRPSAA	MRKLQAT	MKRFNEN	225 YKKLTDP
AKRLQVN	AKRPSAA	LRKPNVA	170 MKRFNEN	IKKFTFG
MHRLQLS	LHHLQPF	115 PHHVSPQ	MKRFNEN	LHHFNLG
MHRLQLS	60 WRRLGVQ	PHHVSPQ	ARRFSIN	FRKVTLT
5 MHRLQLS	FKKISEG	PHHVSPQ	ARRYGDV	PRHLSLA
PKKMSVL	AKKVGYT	PHHVSPQ	LRKAQLQ	230 IHHWTPP
LRRLSAV	AKKVGYT	PHHVSPQ	175 PKHVGFA	PRKAGVF
LRHPNIV	AKKVGYT	120 LRRPSDQ	PKHVGFA	PRKAGVF
LRHPNIV	65 VRRAQLV	FHKVTVQ	PKHVGFA	LRRIQPP
10 YRRMSLA	LRKIQFN	FHKVTVQ	IRRFSLM	LRRIQPP
LRHPNIV	PHHAGLN	FKKATVT	ARRYGDV	235 AKRLTES
IKHFGML	LKKYGDI	PHHIQVA	180 FKKLGIP	AHRVSAL
IKHFGML	LKKFNEL	125 PKKMSVL	FKRLGIP	AHRVSAL
PKRVGLI	70 AKRLTLG	PKKMSVL	FKKLGIP	VHKPGPI
15 PKRVGLI	PKRWSVI	WKKYQFP	FKKLGIP	MRKYQMT
LKHPNVI	PKRWSVI	WKKYQFP	PKHVGFP	240 MRKYQMT
LRRMQEM	PKRWSVI	MHHLTEN	185 IRRFSLM	LHKPQES
LHHFNLG	PKRWSVI	130 MHHLTEN	IRRFSLM	FRKLQDA
LRHVQLA	75 LKKATAY	MHHLTEN	FKKLGIP	YRKYSDY
20 LRHVQLA	LKKATAY	MHHLTEN	MKRFNEN	YRKYSDY
LKRYSEM	LKKATAY	MHHLTEN	FKKLGIP	245 YRKYSDY
LKRYSEM	LKKATAY	MHHLTEN	190 MKRFNEN	LHRAGLL
LKRYSEM	LKKATAY	135 LHRLSLP	MKRFNEN	LHRAGLL
LKRYSEM	80 LKKATAY	LHRLSLP	MKRFNEN	LRKLGLG
25 LKRYSEM	LKKATAY	LKRPSVL	MKRFNEN	AKRAQLQ
FKKPSAA	YRRLQPS	LKHVGPG	IRRFSLM	250 IHKYNAY
LRHVQLA	IKKITAI	LKHYGPG	195 IRRFSLM	IHKYNAY
LRHVQLA	IKKITAI	140 LKHYGPG	IRRFSLM	LRRFGPP
YRHFNAS	85 IKKITAI	LKHYGIG	PKHVGFA	LRRFGPP
30 LHHLQEQ	IKKITAI	VRKYSES	PKHVGFA	VRHISPT
LHHLQEQ	MKHLTPS	AKKITFS	PKHVGFA	255 AKRAQLQ
YHKYSLI	MKHLTPS	LKKIGDT	200 PKHVGFA	VRRASDP
LHHMSLQ	IHKLQDW	145 LKKIGDT	LRRFSVT	PRRLSES
VRKFQIP	90 IHKLQDW	LKKIGDT	LRRFSVT	LHRAGLL
35 AKKIGFG	IHKLQDW	ARRVTFS	IRRFSLT	LHRAGLL
LHRLGII	IHKLQDW	LHHIGMQ	IRRFSLT	260 WRRPSPF
AKKIGFG	VKKISAA	LHHIGMQ	205 FHRVGPI	LRKATIP
WKKYQFP	VKKISAA	150 IRKFSIV	LRRLGMI	MRKATAS
FRKFSPF	95 WHKLGIS	IRKFSIV	LRHLGIV	MRKATAS
40 LRKLNPP	VKKISAA	IRKFSIV	VHKPGPI	YRHWQIP
LRKLNPP	VKKISAA	IRKFSIV	LKRATVA	265 YRHWQIP
VKRLTYP	ARKIGVL	IRRFSLM	210 IRKASDV	MKRFNPP
FRKAQIG	ARKIGVL	155 MRKINPL	ARRAQLA	LRRFNAG
FRKAQIG	100 ARKIGVL	MRKINPL	FRRIQDP	LRRFNAG
45 VRRAQLV	ARKIGVL	MRKINPL	ARRAQEL	ARRAQEL
LRKIQFN	ARKIGVL	PRRYSDY	ARRAQEL	270 ARRAQEL
LRKIQFN	LRHPTWP	VKHLSAS	215 LHRLSLP	ARRAQEL
LRKIQFN	LKKPQDS	160 VKHLSAS	FHKVQVN	ARRAQEL
IRRFQEG	105 LKKPQDS	VKHLSAS	FRKMTEA	LRRFNAG
50 IRRFQEG	LKKFTEY	MRKAGIF	AKRIQLS	VRKIGEL
FKRLNLV	WKKVTVT	PRRYSDY	PKRLTDL	275 LKRMGMS
PRRINLT	WKRYGAL	PRRYSDY	220 FHHANFP	LKRMGMS
PRRINLT	WKRYGAL	165 VKHLSAS	MKKLSYI	ARKIQVP
ARRIQDP	110 PKKINLN	ARRFSIN	MKKLSYI	VKRITES
55 IKKPGVV	WKRFSVP	ARRFSIN	MRKATAS	ARKPGYI
IKKPGVV	MRKLQAT	MKRFNEN	YKKLTDP	280 ARKPGYI

CA 02290722 1999-12-08

FRRLNFA
FRRLNFA
FRRLNFA
FRRLNFA
5 LHRVTIA
FHKATAN
LKRMGMA
PHRASDG
LRRFSIW
10 IKKIGYN
YHHPNDM
LRRLGVT
YHKPSVF
VHKLTIA
15 VHKLTIA
VHKLTIA
VHKLTIA
VHKLTIA
VHKLTIA
20 VKRFSPM
IKKIGYN
IKKIGYN
IKKIGYN
IKKLQIQ
25 IKKIGYN
IKKLQIQ
IKKIGYN
VRRANEA
LRRYNIP
30 LRRYNIP
LRRYNIP
LRKLQEL
LRKLQEL
MRHLTAS
35 VRRYQVL
VRRYQVL
VRRYQVL
VRRYQVL
YHKVTAA
40 YHKVTAA
VRHYNYT
YHKVTAA
YHKVTAA
YHKVTAA
45 PHRYNIL
PHRYNIL
LKRMNPN
LKRLQAN
AKKMGLV
50 VHKLSIN
IKKIGVV
IHRLTIG
VRKANDI
ARRWGIQ
55 ARRWGIQ
PRRLSDS
PHKINPL
ARRWGIQ
LKKLQEA
60 WKHLSDI
WKHLSDI
AKKLSMY
AKKLSMY
AKKLSMY
65 LKKLQIY
VRRLQAL
ARRWGIQ
ARRWGIQ
ARRWGIQ
70 IRRLSPA
LRHITVV
LRHITVV
YKKFGAT
FRKLGLY
75 WKRASVV
WKRASVV
LRHLNFT
PHRASDG
LRRLTPS
80 LKHISEL
LKHISEL
LKHISEL
LKHISEL
LKHISEL
85 LKHISEL
IKKAGDG
FRKLSFT
IKKITAI
IKKITAI
90 MKHLTPS
MKHLTPS
MKHLTPS
MKHLTPS
IKKFQFL
95 VKKLGDF
AHHYSVA
IRRPSPF
IRRPSPF
LRKMGAM
100 LRKAQLQ
LRKAQLQ
LRKAQLQ
LRKLQEL
LRKLQEL
105 LRKLQEL
LRKAQLQ
VKKYQAV
VKKYQAV
AKHLTPV
110 AKHLTPV
LHKVTYL
LHKVTYL
LHKVTYL
WKRVSDI
115 ARKLQDV
ARKLQDV
LRKMGAP
LRKMGAP
LRKMGAP
120 LRKMGAP
LRKMGAP
LRKMGAP
WHKLGIS
YRKLGVY
125 PRRVSEA
PRRVSEA
ARRLTVS
PRKLTLM
PRKLTLM
130 PRKLTLM
PRKLTLM
PHKINPL
LKRFTWT
LKRFTWT
135 ARHATLS
WKRPQMS
VRHFQFL
YRRPSVA
VKRASLL
140 LRHLGLS
LRHLGLS
PHRFQYP
VRKAGIA
VRKAGIA
145 VRKAGIA
VRKAGIA
VRKAGIA
VRKAGIA
LRHLGLS
150 ARKLQDV
ARHATLS
MKHPQFL
VKRVNIL
FKRLGAG
155 MKRVGFQ
MKRVGFQ
MKRLTAG
MKRLTAG
MRHVSIS
160 FRKLSDS
YRKAGLP
YRKAGLP
YRKAGLP
PRKASVG
165 IKKAGAA
PRHISVA
PRHISVA
VKKLNEI
LKKAGYV
170 LKKISIP
LRRVTDL
YRKAGLP
YRRYQDW
YRRYQDW
175 PRRWSWI
LHKIGAL
LRKVSVS
LRKVSVS
LKRLNLA
180 LKHLSLL
MKKLSDL
LHKIGAL
YKRFQLL
YKRFQLL
185 YKRFQLL
AKRFSPP
LKKLGVN
LKKLGVN
AKKVGEI
190 AKKVGEI
AKKVGEI
PKRVQVI
PKRVQVI
ARHLQEY
195 ARHLQEY
FKKLGIP
LHRLQVS
LHRLQVS
LHRLQVS
200 VKKVQAF
VRRLTPS
VRRLTPS
VRRLTPS
VRRLTPS
205 VHRYGES
MKRVGFQ
MKRVGFQ
MKRVGFQ
MKRVGFQ
210 PRKVGYW
IKKAGAA
IKKAGAA
IKKAGAA
IKKAGAA
215 PRKIGYW
WKKIGIW
AKHVGYS
AKHVGYS
AKHVGYS
220 AKHVGYS
MRRPNFQ
MRRPNFQ
AKHVGYS
AKHVGYS
225 IKKAGAA
IKKAGAA
IKKAGAA
IKKAGAA
AKKFGVL
230 FKHPQPF
FHKPGEN
LKKISAY
VKKIQPL
AKRLQDY
235 AKRLQDY
AKRLQDY
VKHLSLG
VKHLSLG
VKHLSLG
240 VKHLSLG
VKHLSLG
VKHLSLG
VKHLSLG
VKHLSLG
245 VKHLSLG
VKHLSLG
VKHLSLG
VKHLSLG
LRRMSLI
250 VKRINMA
VKRINMA
VKRINMA
LRRMSVI
IRRIGLF
255 IRRIGLF
ARHLTLS
ARHLTLS
ARHLTLS
AHHFSEP
260 AHHFSEP
AHHFSEP
AHHFSEP
AHHFSEP
AHHFSEP
265 VHRFTVP
VHRFTVP
VHRFTVP
AHHFSEP
AHHFSEP
270 AHHFSEP
VHRFTVP
VHRFTVP
VHRFTVP
VHRFTVP
275 VHRFTVP
VHRFTVP
AHHFSEP
AHHFSEP
IRKYNLS
280 PRRPGPT

CA 02290722 1999-12-08

	PRRPGPT		VKKINES		IHRLTEA		IRKYGLN	225	IKKFGLT
	YKRWQDV		AKRVTIM		AKKAGAA	170	LKHLNLG		IKKFGLT
	YKRWQDV		AKRVTIM	115	PRHLQLA		PRRLTAT		FRRITAA
	YKRWQDV	60	IRKFQIL		PRHLQLA		PHKINPL		FRRITAA
5	YKRWQDV		MHKPGLW		PRHLQLA		VRRLQAL		FRKVGDA
	YKRWQDV		LKKFNEP		PRHLQLA		IRHVSFS	230	LKRFNDP
	YKRWQDV		LKKFNEP		PRHLQLA	175	AKRLQEA		ARRPTPN
	YKRWQDV		LKKFNEP	120	PRHLQLA		AKRLQEA		YHKVNFS
	YKRWQDV	65	WKKVTVT		PRHLQLA		AKRLQEA		ARHPGDY
10	YKRLQDS		WKKVTVT		PRHLQLA		YKHINEV		IKRFSAS
	LKHLTLA		FRKLGLY		PRHLQLA		LKKFNEP	235	LRKLSAV
	FRKLQLS		IRRLQLY		PRHLQLA	180	LKKFNEP		LRKLSAV
	MRKFQEQ		VKKPGES	125	PRHLQLA		LKKFNEP		LRRVQDL
	AHKIQVQ	70	MKKVGVT		PRHLQLA		AHRIQEL		AHRVTDS
15	LKHLTLA		AKRIGEV		AKRVTIM		LRRPSDQ		AHRVTDS
	LKHLTLA		AKRIGEV		AKRVTIM		PRKVSEL	240	MRRISLF
	LKHLTLA		ARRLSFI		AKRVTIM	185	FHHPSAV		MRRISLF
	LKHLTLA		LKKLGIL	130	AKRVTIM		LRRASAY		MRRISLF
	LKHLTLA	75	LKRISIA		AKRVTIM		PRKLQAA		MRRISPF
20	IRKYNLS		LRKPSLQ		AKRVTIM		PRKLQAA		FRRITLY
	MKRLTAG		LRKPSLQ		AKRVTIM		VKRFSPI	245	MRRISPF
	VKRLGIP		WHRVTAL		AKRVTIM	190	MRKISLF		FRRITLY
	AHHFSEP		WHRVTAL	135	AKRVTIM		MRKISLF		VKKPGAS
	ARHPSPS	80	WHRVTAL		AKRVTIM		WHRVTAL		MKKPGES
25	VRRPSES		AHRLSAS		AKRVTIM		WHRVTAL		VKKPGAS
	PRHLQLL		AHRLSAS		AKRVTIM		WHRVTAL	250	VKKPGAS
	IKHFQII		VRKPGAS		AKRYTIM	195	WHRVTAL		VKKPGAS
	IKHFQII		LHKPGVY	140	PRRAGPI		AKRISAY		VKKPGAS
	PRHLQYV	85	PHKPQFT		PRRAGPI		ARKLSEL		VKKPGAS
30	PRHLQYV		PHKPQFT		VRRVGDP		ARKLSEL		VKKPGAS
	LKHLSLL		LKRYSIP		VRRVGDP		ARKLSEL	255	VKKPGAS
	FKRLQPT		WKRASVV		AKRAGVT	200	ARKLSEL		LKKPGAS
	LKKVGFN		PHHPNPP	145	YHKVNFS		LRRMQEM		VKKPGAS
	YKHLGVF	90	PHHPNPP		AKKLGEM		IRKLQIQ		VKKPGAT
35	FKRIGVP		PHHPNPP		AKKLGEM		FHKWSLS		VKKPGAS
	YRKVQPS		LKRWSAA		AKKLGEM		AKKVGEI	260	VKKPGAS
	YRKVQPS		LHKPGVY		AKKLGEM	205	AKKVGEI		VKKPGES
	YRKVQPS		PRHLQLL	150	AKKLGEM		AKKVGEI		VKKPGAS
	PRKISYS	95	AKKLGEM		FHRASVL		VRRVSVA		VKKPGAS
40	PRKISYS		YKKVQLV		FHRASVL		AKRVGDT		VKKPGAS
	PRKISYS		LKKMQAN		LRKLGLG		FHRASVL	265	ARRANLI
	PRKISYS		WKRATYI		AKRLQEA	210	FHRASVL		VRKPGAS
	PRKISYS		WKRATYI	155	ARRVNLT		MRRISPF		VKKPGAS
	ARKLTAQ	100	WKRATYI		AKRLQEA		PKKATEL		VKKPGAS
45	YRKVQPS		VRKVGYL		AKRLQEA		ARHLTLS		VKKPGES
	LKKLGIQ		PRHPNVF		PRRPGFW		ARHLTLS	270	VKKPGAS
	PRKINVS		YKRWQDV		AKRLQEA	215	ARHLTLS		VKKPGAS
	IRRLQLY		YKRWQDV	160	LKHVNAL		PRHISVA		VKKPGAS
	LKKFSLG	105	YKRWQDV		LRKLNLS		PRHISVA		VKKPGAS
50	VKRLTDA		PRHLQLA		LRKLNLS		LHRINLT		VKKPGAS
	VKRLTDA		PRHLQLA		LRKLNLS		MRKLQAT	275	VKKPGAS
	LRRPGLG		PRHLQLA		LRKLNLS	220	LKRMNPN		VKKPGAS
	LRRPGLG		FHKYGEY	165	IKRLQIA		WKRITIQ		VKKPGAS
	ARHATLS	110	AKKAGAA		IKRLQIA		WKRITIQ		VKKPGAS
55	AKKAQAQ		AKKAGAA		FHHLTYL		WKRITIQ		VKKPGAS
	AKKAQAQ		AKKAGAA		FHHLTYL		LRRLGLV	280	VKKPGAS

CA 02290722 1999-12-08

VKKPGES
VKKPGAS
VKKPGES
VKKPGES
5 VKKPGVS
LKKPGAS
LKKPGAS
VKKLNEI
VKKPGAS
10 VKKPGAS
LHKVTYL
VKKPGAS
VKKPGAS
VKKPGAS
15 VKKPGAS
VKKPGES
VKKPGAS
VKKPGAS
MKRFNEN
20 MKRFNEN
ARRAQEA
LKRIQIW
P
ARHPTYY
25 ARHPTYY
VKKPGAS
VKKPGAS
VKKPGES
VKKPGES
30 VKKPGES
VKKPGES
VKKPGES
VKKPGES
LKKPGAS
35 IKKPGAS
VKRPGAS
VKKPGAS
VKKPGAS
VKKPGAS
40 VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
45 VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
50 VKKPGAS
LKKPGAS
VKKPGAS
LKKPGAS
LKKPGAS
55 VKKPGAS
VKKPGAS
VKKPGAA
VKKPGAS
VKKPGAS
60 VKKPGAS
VKKPGAS
LKKPGAS
LKKPGAS
LKKPGAS
65 LKKPGAS
LKKPGAS
LKKPGAS
LKKPGAS
LKKPGAS
70 LKKPGAS
LKKPGAS
AHRPGIA
VKKPGAS
IKKPGAS
75 VKKPGES
VKKPGES
VKKPGES
VKRPGES
VKKPGAS
80 PRRPSLT
VKKPGES
VKKPGES
VKKPGAS
VKKPGES
85 VKKPGES
VKKPGES
AKKPGES
ARHLGYS
LKKPGAS
90 VRKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
95 VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
100 VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
105 VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
VKKPGAS
110 VKKPGAS
LKKPGAS
VKKPGAS
VRKPGAS
VKKPGAS
115 VKKPGAP
LKKPGAS
VKKPGAS
VKKPGES
LKKPGAS
120 LKKPGAS
LKKPGAS
LKKPGAS
ARHPSMV
VKKPGAS
125 VKKPGAS
VKKPGAS
VKKPGAS
LHRYGYN
VKKPGAS
130 VKKPGES
VKKPGAS
VKKPGAS
VKKPGAS
VRRIQEP
135 VRRIQEP
VRRIQEP
VRRIQEP
VKKPGAS
MKKPGAS
140 LKKPGAS
VKKPGES
VKKPGES
VKKPGES
VKKPGES
145 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
150 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
155 VKKPGES
VKKPGES
VKKPGES
AKRPGES
VKKPGES
160 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
165 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
170 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
175 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
180 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
185 VKKPGES
VKKPGES
VKKPGES
VKKPGES
VKKPGES
190 VKKPGES
VKKPGAS
ARRPGVL
VKKPGES
VKKPGDS
195 VKKPGEA
VKKPGAS
LRRATEY
ARRFGVP
ARRFGVP
200 ARRFGVP
ARRFGVP
VHRFQEY
VHRFQEY
VHRFQEY
205 PHRPSLI
VKRLTDA
VKRLTDA
FRHLSFY
PRRPQEP
210 PRRPQEP
PRRPQEP
PRRPQEP
LRHFTEY
VRKVGYL
215 YRKLGVY
VRHPNYL
VKKFGEN
IRKYGDP
MHRYQVN
220 VKKFGEN
IRKYGDP
LHRLNIP
LHRLNIP
LHRLNIP
225 LKKYQIF
MRRISPF
MRRISPF
FRRITLY
PKKISEL
230 PKKISEL
MRRISPF
MRRISPF
MRKISLF
FRKAQIG
235 FRKAQIG
MRRISLF
MRRISLF
FRRITLY
MRRISPF
240 FRKAQIG
MRRISPF
IKKINPL
IKKINPL
FRKAQIG
245 PKKATEL
PKKATEL
PKKATEL
PKKATEL
LHKLSES
250 LHKLSES
AHRAGVL
LRRLQLL
LRRLQLL
PHKVSAG
255 PHKVSAG
PKKATEL
PKKATEL
PKKATEL
WRHPTMG
260 WRHPTMG
WRHPTMG
WRHPTMG
WRHPTMG
PKKATEL
265 WRHPTMG
PKKATEL
PHKVSAG
FKKPSPF
LHKLSES
270 FRHPNIV
FRHPNIV
FRKLQEI
AKKLQPI
AKKLQPI
275 MRRYTLN
MRRYTLN
VHRLSAT
IKKAGAA
VRKVTLS
280 VRKVTLS

VRKVTLS
VRKVTLS
IKKFTFG
IKKFTFG
5 IKKFTFG
IKKFTFG
MHRYQVN
AKKLQFY
AKKLQFY
10 AKKLQFY
MKKLQPT
MKKLQPT
MKKLQPT
MKKLQPT
15 MKKLQPT
MKKLQPT
MKKLQPT
MKKLQPT
MKKLQPT
20 MKKLQPT
LRHLGAV
LRHLGAV
LRHLGAV
FHKIGVG
25 YHKIGVG
YHKIGVG
YHKIGVG
YHKIGVG
WRRFSDQ
30 AKHLGFQ
AKHLGFQ
AKHLGFQ
AKKPGLA
ARHISFG
35 LHHVGWF
LKHIQAP
LHHATVL
LHHATVL
LKKLTEL
40 PRHINLP
MKKYQEQ
AKRPNYV
AKRPNYV
IHRYGEG
45 LRHYGYL
LRHYGYL
IHKLSVS
VKRPQLM
VKRPQLM
50 VKRPQLM
VRKYQAQ
LRRATVL
LRRATVL
LRRLNFV
55 IRKPGES
LRKYTVG
AKRLQLL
YRKPSPQ
AHKMQLP
60 LKKVTEQ
LRRMQEM
MRKYTVL
LKKASVI
ARHYSLS
65 PRHFGPV
AKHASDN
FRRPSEG
FRRPSEG
PRHPQVA
70 AKKIQVP
IHHPGAF
LKRPTAL
FRHAQDL
PKKAQLI
75 IKKISPF
ARKLQDV
AHHISLA
AHHISLA
IRRAGPG
80 PRKASLQ
LHHVNFS
LKRLNPQ
PRRFSAL
PRRFSAL
85 PRRFSAL
IRKIGDT
VRHPNYL
IRKIGDT
MKRLSYI
90 MKRLSYI
PHKVSPY
WHRITVI
WHRITVI
WHRITVI
95 WHRITVI
WHKVTAN
WHRITVI
WHRITVI
WHRITVI
100 WHRITVI
WHRITVI
WHRITVI
MKRLSYI
WHKVTAN
105 VKKLNEI
WRHLTPT
FKKAGWT
FKKAGWT
LRRITYV
110 YKRLNLT
YKRLNLT
PKKLGPS
VRRLTVG
VHHPSFL
115 LRRLSAV
LHHWSWA
LHHWSWA
MHRYQVN
MRRISPF
120 MRRISPF
IKRMQEV
IKRMQEV
IKRMQEV
IKRMQEV
125 IKRMQEV
IKRMQEV
FKHITPL
LHKAGDI
YRRFSVQ
130 LKRVQLY
LKRVQLY
ARKLSVI
PRRWSPI
VHKWSEL
135 YRRVSDM
AKRAGVT
AKRAGVT
AKKLTDI
LHKVTYL
140 LHKVTYL
LHHVNFS
MRRYTLN
YKRLNLT
YKRLNLT
145 LRRIQPP
LRRIQPP
LRRANPS
LRRANPS
LRRIQPP
150 LRRIQPP
FKRFNAL
FKRFNAL
IRKPQVV
FRRLSDQ
155 PRRFSAL
PRRFSAL
PRRFSAL
FHKPSYF
LRRVTIA
160 VKRLGAG
VKRLGAG
IRKASDV
PKRAGIM
PHKPQFT
165 IRKMSLL
VRHATAA
MKKLQPT
MKKLQPT
LHHISLL
170 ARHPGDY
MRHLSPT
MRHLSPT
LHKVNFY
LRKITIG
175 LRKITIG
ARRITEV
ARRITEV
VKRVGII
VKRVGIS
180 IRKYSII
WRHVQPT
PRKAGDF
IKKLTLQ
LKRMTII
185 WHKVTAN
IRKWNVT
IRKWNVT
LRHLGLQ
LRHLGLQ
190 MRRLGVV
MRRLGVV
MRRLGVV
MRRLGVV
MRRLGVV
195 MRRLGVV
MRRLGVV
MRRLGVV
MRRLGLA
MRRLGLA
200 MRRLGLA
MRRLGLA
LRRLGLG
LRRLGLG
WKKAGLN
205 PRHVSPV
VKKLGFN
VKKLGFN
VKKLGFN
VKKLGFN
210 FHHMNFI
LKRLGMW
LKRLQPG
LKRLQPG
ARRLQLS
215 PKKPTAI
PKKPTAI
YHKYSLI
WRRFSDQ
LKKAGLS
220 AKRLNVT
AKRLNVT
AKRLNVT
AKRLNVT
VKRISAT
225 VRRPQAV
AKHFSAL
AKHFSAL
YKHINEV
YKHINEV
230 IKKLTLQ
LRHVSVY
VRRLTVG
PRRYSEG
LHKIQEQ
235 VKKVQAA
VKKVQAA
LRRMQEM
PRRINLT
IRRLSLG
240 YHKYSLI
YHKYSLI
AHRMGMG
LKKINEL
AKRLQEA
245 AKRLQEA
IRRYSDA
IRRYSDA
IRRYSDA
PRRLQEG
250 AHKVTPV
VKKPGAS
VKKPGAS
VKKPGAS
LRRLGAW
255 LRHINVT
LRHINVT
LRHPQPG
LRHPQPG
AKKVGYT
260 LKKINEA
IRRIGLF
IRRIGLF
LRHLGEV
AKHLNVQ
265 PKRWSVI
PKRWSVI
LKKATAY
LKKVNEA
LKKVNEA
270 IHHLTAL
IRKPSPF
AKKIGEL
AKKIGEL
VRRAGFA
275 AKKIGEL
LKKVQPW
VRHLTDW
VRHLTDW
VRHLTDW
280 VRHLTDW

CA 02290722 1999-12-08

IRRAGYP
FRRLSLL
VRRAGYA
VRRAGFA
IRRAGYP
VHRLGYV
VHRLGYV
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
VHRLGYV
VHRLGYV
LHHLSVL
AKKIGEL
AKKIGEL
MRRLSLS
WKRVNEP
WKRVNEP
VRRASEP
VRRASEP
IRKYSAI
PRHISVA
LKKAGYV
IHHINEI
VHKANIM
LRHMGLF
LRHMGLF
VHKANIM
AKRIQIS
AKRIQIS
VKKLTVN
VHKANIM
MHRFGPI
YRKYTEQ
YRKYTEQ
ARKASIV
LHHMSLQ
PHHWQPS
LKRLSEF
YRKVQEL
YRKVQEL
AKKAGEI
AKKAGEI
AKKAGEI
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
FKKLTDS
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
MRKATPT
WHKLSLA
LKKYTEY
PKHLTDA
IKRPQDQ
IKRPQDQ
ARRLGEA
PHHPNPP
PHHPNPP
PHHPNPP
PHHPNPP
LRKVSVS
LRKVSVS
LRKVSVS
LRKVSVS
LRKVSVS
LRKVSVS
LRKVSVS
LRKVSVS
MKRPSVV
FRHMSLL
FRHMSLL
FRHMSLL
LHKIQEQ
VKRFSPI
IKRWQAI
WKRPTEI
YRRFTEI
IKKPQAQ
PHHIGVA
IHHAQDL
FRKLSFT
YRRVTET
IRKYSII
MKRLSYI
AKRIQIS
AKRIQIS
MKRPSVV
MKRPSVV
MKRPSVV
MKRPSVV
FKKLGIP
IHRVSAA
IHRVSAA
LKHPNIV
WRKANLT
PHRYNIL
PHRYNIL
PHRYNIL
PHRYNIL
WRRWNFI
PRHASLP
FHHLSVV
FHHLSVV
FHHLSVV
LKKATEY
LKKATEY
LKKATEY
LKKATEY
LRKPSYA
PRRLQLA
AKKASAF
AKKASAF
AHRLNIA
AKKLGEM
LKKANLQ
LKKANLQ
LRKLQAQ
LRKLQAQ
LKRLQLQ
LKRLQLQ
FHKATAN
LKHMSPP
LKHMSPP
LKHMSPP
LKHMSPP
AKRITEW
PRRISAV
PHHIGVS
PHHITVS
PHHIGVT
PHHIGVT
PHHIGVT
PHHIGVT
PHHITVS
PHHITVS
PHHIGVT
FRKLSFT
AKRVGLP
AKKPTAA
LKHLQDI
LRRASMQ
LRRASMQ
LRRASMQ
LRRASMQ
YKKVQLV
LKRLQMQ
LKRFQFV
LKRFQFV
IKKIGYN
IKKIGYN
FKKATVT
IKHLNVS
FHKMGVG
FKKATVT
ARHPQVS
AKRVGLP
YKKLSLI
PRRATIS
PRRATIS
AKKIGLV
ARKLQDV
PKKAQLI
PKKAQLI
ARKWGYT
ARKWGYT
ARKWGYT
ARKWGYT
LRKLQLQ
IKRPQDQ
IKRPQDQ
VRKLQAN
VRKLQAN
YHHLTYI
PRHPSFP
WRKLGAP
WRKLGAP
MKHLSIA
MKHLSIA
IKRLNVQ
IKKFGLT
IKKFGLT
LKRLQDV
LKRLQDV
PKKASFQ
PKKASFQ
PRKLQAA
PRKLQAA
LRRLQDV
LRRLQDV
LRRLQDV
VRKIGEL
IKHPNLV
IKHPNLV
LKRANEF
PRKFNET
PRKFNET
FKHLNEI
PHHVSPQ
PHHVSPQ
PHHVSPQ
PHHVSPQ
LKKLNLS
LKKLNLS
PKHVGFA
PKHVGFA
PKHVGFA
PKHVGFA
VRRLGIP
VRRLGIP
AKRASVF
AKRASVF
IHHAQDL
YRRATVF
VKKMTFS
VKKMTFS
LRRVTAA
LRRVTAA
LKKATAY
LKKATAY
PRKLQAA
PRKLQAA
AKKPTAA
IHHAQDL
PRKLQAA
PRKLQAA
AKKWTAV
AKKWTAV
VHRPQPL
YHKPSVF
YHKPSVF
LRKASVT
LRHVNEQ
LRHLNFT
LHRAQAQ
LHRAQAQ
LHRAQAQ
LHRAQAQ
LKKINYQ
VHRPQPL
VHRPQPL
VHRPQPL
VHRPQPL
FKKATVT
PKKPSPT
VHKYNPT
IKKVQDL
IKKVQDL
IKKVQDL

CA 02290722 1999-12-08

PHHFNAS
PHHFNAS
PHHFNAS
YHRINEG
YHRINEG
YRHFQIP
LRRLQEG
YHRINEG
FKHITPL
FKHITPL
FKHITPL
FKHITPL
LRRLQEG
LRRLQEG
WKKAGLN
WKKAGLN
WKKAGLN
AKKLNVQ
IKRLNVQ
AKKLNVQ
YKKPGYN
ARHPQEQ
VHRLSAT
IKKLSMT
ARKYQYW
WKKAGLN
WKKAGLN
MHHLTEN
MHHLTEN
MHHLTEN
AKRYQEF
AKRYQEF
AKRYQEF
AKRYQEF
AKRYQEF
AKRYQEF
FKHFQEF
VKRYGPM
MRRYTLN
FRRLSIS
FRRLSIS
FRRLSIS
FRRLSIS
YRHIGVL
LHKLNIV
YRHIGVL
LRHPNIV
VKKMTFS
VKKMTFS
VKKMTFS
VKKMTFS
VKKMTFS
VKKMTFS
VKKMTFS
VKKMTFS
AKKLQPA
AKKLQPA
AKKLQPA
LRRPQLP
AKKLQPA
LRKIQES
YHRPGEG
YHRPGEG
PHHLTVI
PHHLTVI
PHHLTVI
VKRPGAA
FKKATVT
FKKATVT
MKKVTIS
FKKATVT
MKKVTIS
FKKATVT
VKKPGAS
LRKASDS
LRKASDS
LRKASDS
LRKASDS
MKKITLL
MKKITLL
VKKLTVN
VKKLTVN
VKKLTVN
VKKLTVN
LRRLSAG
LRRLSAG
LRRLSAG
AKRYNVS
AKRYNVG
IHKYNAY
PHHYTPI
PHHYTPI
AKKATVN
LKKAQDV
VKKWGIN
LKKLGLI
VRRLQAA
VRRLQAA
YHKIGVG
PHRLGAM
IKRATLN
FKHITPL
VHHVTVS
PRRYTIA
PRRYTIA
PRRYTIA
PRRYTIA
PRRYTIA
PRRYTIA
LHHLQEQ
ARKWGYT
AHHFSEP
LHKFNEF
LHKFNEF
LHKFNEF
LHKFNEF
LHKFNEF
MKKLQES
IKRPGAS
FRRFQMI
FRRFQMI
PRRWSWI
AHHFSEP
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
IRHFTFT
AKKLQPA
AKKLQPA
PKKPSAG
AKRPGIY
AKRPGIY
IRHPQYN
MKHLTPS
MKHLTPS
LRRLGAW
LKKPTVN
LKKPTVN
LKKPTVN
IRKWNVT
IRKWNVT
AKRPGIY
IRRMNEN
FKKPTPS
FKKPTPS
ARHLQPG
PKKASFQ
PKKASFQ
LRRATIS
FHKYTIG
FHKYTIG
FHKYTIG
FHKYTIG
MKHPQEM
IKRWQAI
FRRLTPT
LHKLNAV
PRRLSLG
PRRLSLG
IKKISPF
MKHPNIV
MKKITLL
MKKITLL
MKKITLL
MKKITLL
WRRPGAA
WRRPGAA
WRRPGAA
WRRPGAA
LKHPNIV
MKKLQPT
MKKLQPT
MKKLQPT
LHHPGVV
VHKPGPI
ARHPQLL
VKRAGLN
PRKIQFT
AKKLQPA
AHRPGWL
AHRPGWL
AHRPGWL
AHRPGWL
AHRPGWL
IKKISPF
VRKASLS
VRKASLS
AHKVGIT
AHKVGIT
IHHISEP
IHHISEP
IHHISEP
IHHISEP
IHHISEP
IHHISEP
IHHISEP
IHHISEP
IHHISEP
LKKYGVT
LKKYGVT
LKKYGVT
VRHVTIQ
IHHISEP
IHHISEP
IHHISEP
LRRLNFQ
WHRPTEL
LKKYGVT
LKKYGVT
VRHFQFL
MRRINFQ
AKKIGPL
IHHISEP
IHHISEP
IHHISEP
MKKMQLP
LRRLGAA
FRKMTLF
IHHPGYL
VKHANET
LKKYGVT
LRRLNFQ
MKKLSYI
MKKLSYI
LKKPGAA
LKKPGAA
IKHLNVS
IKHPNLV
IKHPNLV
IKHPNLV
IKHPNLV
ARRVNLS
PRRYSPV
IKRLQVA
IKRLQVA
IRHFTFT
LKKYGVT
MRRINFQ
IKRIQAM
IKRIQAM
IKRIQAM
FHKVTVQ
LRRLTPS
MRRLNYQ
FKKATVT
PRRWSWI
ARKLQDV
PHKFTVS
YKKVQIQ
LKRAGIS
LKKATEY
VKKPGAS
LRKIQFN
ARRIQDP
ARRIQDP
FKRPTPP
FKRPTPP
FKRPTPP
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LRRPTET
LHHFNLG
VRRIQEP
ARHLQEN
FKRLGAG
AHRLQAV
IHRWTIL
MHKAQLV

CA 02290722 1999-12-08

YRHFQIP
LKRLSLS
WRRLQEL
WRRLQEL
5 WRRLQEL
PRRASWV
FKHYQMS
FKHYQMS
VRKASDV
10 VRKASDV
VRKASDV
VRKASDV
IRKASDV
VRKASDV
15 VRKASDV
VRKASDV
VRKASDV
YKRFQLL
LRRITLP
20 VKKLQLM
IHKPGYL
LKKLTEL
IHKFGFS
IRKFQEQ
25 YRRMSLA
LRRLTTL
LRRLQDV
LRRLQDV
LRRLQDV
30 MRKISLF
MRRISPF
MRKISLF
MRRISLF
MRRISPF
35 FRRITLY
MRRISPF
FRRITLY
MRRISPF
ARHWGDG
40 ARHWGDG
VRHFQFL
MKKVGVT
VKHIGEL
VRHFQFL
45 VRHFQFL
LRRLGVT
LKRANVQ
VKHIGEL
PRRLNVA
50 PRRLNVA
LKHLSPT
LKHLSPT
MKRLTLG
MKRLTLG
55 MKRLTLG
FRHAQLL
FRHAQLL
LKHMSVS
IRRLSLG
60 IRRLSLG
ARKFNVG
ARKFNVG
VKHLNVS
VKHLNVS
65 LKKLQIF
LRKASVT
VHKYGLA
VHKYGLA
VKRWGFG
70 IHHISVM
IHHISVM
IHHISVM
IHHISVM
IHHISVM
75 VKKPGAS
LRKPGAS
LKKIQEL
AKKITAA
PRKVTAA
80 PRKVTAA
IRRASAI
LKKMQAN
LKKMQAN
PKHLTDA
85 IRRMGES
IRKYGLN
VRKITVG
VHKATDT
VHKATDT
90 ARKLGFT
ARKLGFT
PKRLNFS
FKKPSFS
LRRAQDI
95 PRRISAV
LRKVTAN
LRKVTAN
LRKVTAN
LRKVTAN
100 LRKVTAN
LRKVTAN
LRKVTAN
LRKVTAN
LRKVTAN
105 LRKVTAN
AHRLGLI
PRRFSPP
MRRYTIS
VRKFQIP
110 VRKFQIP
AHRPGLQ
AHRPGLQ
FHRIQDQ
VHHPQLV
115 FHKYGEY
VRKYQEQ
AKKVTVT
AKKVTVT
AKKVTVT
120 AKKVTVT
AKKVTVT
AKKVTVT
PKRISPV
MKHASDS
125 IKRATDV
IKRATDV
AHHISLA
AHHISLA
LKRLNPQ
130 IKKLTLQ
AKRASVF
LRHLGLT
WRKAGYQ
LKHYGPG
135 LKHMQFP
WKHLQEN
AHKISYP
AHKISYP
AHKISYP
140 VKRISAT
LKHWGLY
IRKLGWG
IRKLGWG
YHRFSPL
145 PRHFNPV
LRHLGIV
AKRISIN
AKRISIN
MKKITLL
150 MKKITLL
LKHPNLV
LKHPNIV
AKRISAY
LKHPNII
155 LKHANIV
LKHANIV
LKHANIV
LKHANIV
YKHTNEV
160 PKHAGIM
PKHAGIM
VRRVGAP
VRRVGAP
LKHLSLL
165 LRRLSAV
LKRLQLQ
PHRPSLI
PRHLQLL
PHRPSLI
170 PRRMSDP
PKKAGDI
PHRPSLI
PHRPSLI
PHRPSLI
175 MKRYGLL
MKRYGLL
AKHPSVL
LRRFSIL
VRHVTEN
180 LRKATIP
LRKATIP
LRKATIP
IKRLNIQ
FKKVSPW
185 WHHAQLA
VKHFNDV
ARRIQDP
ARRIQDP
VKKIQAS
190 FHHWQIV
ARKLQDV
YHRLSEL
LHRPNLL
LHRPNLL
195 LHRPNLL
LKKLQIY
LHHATIL
LHHATIL
IKKFQDL
200 PKKAGLS
PKKAGLS
PKKAGLS
FRRINMV
VRHIQEF
205 VHKPGPI
IKHPNLV
FKRIGDL
YRKPTVT
AKKVGYT
210 AKKVGYT
FRHPQWT
AKKWNLP
YKRFSEA
PKKLTEW
215 PRHLQLL
WHRISVF
AKKVTVT
AKKVTVT
AKKVTVT
220 VKKLSDV
AKRIGEV
IRKPSPY
IRKPSPY
LKKLSIF
225 LKKLQIY
LKKLQIY
VKKINES
LRKLQAQ
LRKLQAQ
230 LRKLQAQ
LKHASFL
VKKLSDV
VKKLSDV
VKKLSDV
235 AHKWSLP
YRRPSVA
YRRPSVA
YRRPTVA
FRKPSVA
240 PKRLTDL
PKRLTDL
PKKLTPL
VHRYSVL
LRRYNVA
245 VRRAGLV
YKKPGYN
AHRWGIQ
YRRVGDV
VKKLGDF
250 VKKLGDF
VKKLGDF
VRRPTVG
VKKPGAS
IRHYQLL
255 IRHYQLL
LRKPGEI
LRKPGDT
LRKPGDT
LHKAGIL
260 FKKIQAL
FKKIQAL
FRHPGYS
PRRATDW
AKKWTAV
265 LHHAQDY
IKRIQAM
LKHVNAL
LKHVNAL
LKHVNAL
270 LKHVNAL
IRKIGEA
LRHITVV
VKRPGDL
VKRPGDV
275 VKRPGDL
VKKVQEA
LRHVSVY
LRHWSDM
LKRLTFQ
280 VKHLSLQ

VKHLSLQ
LKKPSAL
PRHLGMG
LKKPSAL
5 PRRWSWI
PRRWSWI
LRKPSVQ
IRRLSPA
AHRLQAV
10 LRRLTMN
LRRLTVQ
LKKIQFP
LKKIQFP
LRKAGPS
15 PKHLTDA
AKKIQVP
AKKATVN
LRKVGAP
VKHLSLQ
20 PRRWSWI
LKRLTFQ
LKRLTFQ
VKHLSLQ
LKKPSAL
25 LKKPSAL
LKKPSAL
LKRLTFQ
VKHLSLQ
LRKPSVQ
30 LKRLTFQ
AKHFSLQ
VKHLSLQ
VKHLSLQ
LKKPSAL
35 IRHVGAT
PRRWSWI
PRRWSWI
PRRWSWI
LRKPSVQ
40 LKKPSAL
PRRPGPT
LKKPSAL
VKHLSLQ
YHRPGLG
45 LRRVQYL
MKRLGMN
VRKYGEG
VRKYGEG
LRHPNTL
50 IKKVQDL
LRRLGLV
YKKPSWL
LRKPGIN
LRKPGIN
55 PKRVSDS
IKKIQEA
WHRAQAS
WRRLNVA
LHRLQVA
60 PHHVSLA
PHHVSLA
LKRANEF
PKRVSLV
AKRPSVS
65 PRKPSAS
PRKPSAS
ARHATYG
LRRPSLV
LRRVQYL
70 LRRVQYL
LRRVQYL
AKRPSAA
AKRPSAA
LKKYNLF
75 LKKYNLF
LKKATAY
PKRLGPL
PKRLGPL
PKRLGPL
80 PKRLGPL
YRRLQPS
YRRLQPS
LHRPQVG
VRRFQIA
85 VRRFQIA
FKHASPI
PRRVSLA
PRRVSLA
IRRPGAT
90 LRRLQAQ
PRRYTIA
MKKMGEM
MKKMGEM
PRRLSDS
95 PHHLTVI
PHHLTVI
PHHLTVI
PHHLTVI
PHHLTVI
100 AHRPGWL
AHRPGWL
VRRLGLS
VRRLGLS
PHHLTVI
105 MRRYTLN
LKKLGLI
LKKLGLI
LKKLGLI
LRKANDQ
110 VHHPGVP
MRHLGAF
MRHLGAF
MRHLGAF
AHRIQEL
115 AKRLQEA
AKRLQEA
LKKMSPQ
YHHPNDM
LKKLGVN
120 VKKYSIV
FHKYGEY
VKKASYL
VKKASYL
VKKASYL
125 VKKASYL
PRRIQLS
MKHYTEA
AHKIQVQ
AKRLSFV
130 VRHVSFV
YKRPGYG
IKKLNEI
IRKFSIV
IRKFSIV
135 VKKVQAA
FHRIQDQ
LHHPNTV
LKKPQDS
LKKPQDS
140 PKRPGPW
WRRFSDQ
PRRYTIA
PRRYTIA
PRRYTIA
145 AKRPSAA
AKRPSAA
MKRLNEI
MKRLNEI
MHHWSIP
150 VKKFSIV
IRRYSDA
PKKISDL
PKKISDL
MHRVSVI
155 LRHFSIS
LRHFSIS
PRKMSVI
LHRLGLP
LHRLGLP
160 FKRISEQ
VRRLGLS
VRRLGLS
VRRLGLS
VRRLGLS
165 VRRLGLS
LRKLQEA
LKKFTEY
WKRATYI
PRRLNVA
170 PRRLNVA
FKKIQAL
FKKIQAL
FKKIQAL
FKKIQAL
175 FKKIQAL
FKKIQAL
LHRLGII
MKRFNPP
PRKVSEL
180 PRKVSEL
LHRLTMM
ARHPGDY
PKRPGPA
IHHISEP
185 IHHISEP
MKRFNPP
PKKMTFP
PKKMTFP
LRHLQVI
190 IKKFTFG
LKRFSYN
ARHFSAA
ARHFSAA
VKKFSAM
195 FKKYSFM
FKKYSFM
FRKATPY
FRKATPY
IRHLNAT
200 MKKVQES
MKKVQES
MKKLQES
MKKLQES
IRHLNAT
205 IRHLNAT
VKRLQEQ
LKRFSYT
VKRLSVS
MKHINLS
210 WRKPGPS
VRRVSFA
PKRLSAV
IKRYNLQ
LHRLSDP
215 LKKATFA
LKKATFA
LKKATFA
LKKATFA
LKKATFA
220 LKKATFA
LKKFQVT
VKRLQEQ
PRRAGPG
MKKLSDL
225 MKKLSDL
LKHIQEL
VKHLSLQ
IRKANIP
IRKANIP
230 VKKPGES
WKRPQMS
VKHLQVF
VKHLQVF
LRKISEF
235 WKRPQMS
LRHVSVY
VRHVSVY
VRHVSVY
LRHVSVY
240 LKHLSLL
LKHLSLL
PRKLNFQ
YHKIGLG
LRRIQPP
245 LRRIQPP
MKHLTVQ
MKHLTVQ
LRRIQPP
LRRIQPP
250 LRRIQPP
LRRIQPP
LRRIQPP
LRRIQPP
LRRIQPP
255 LRRIQPP
LRRIQPP
LRRIQPP
LRRIQPP
LRRIQPP
260 LRRIQPP
LRRANPS
LRRANPS
LRRIQPP
IRRMQYF
265 VKKLSDV
ARRVGLV
ARRVGLV
LKKAQMA
LKKAQMA
270 LKKAQMA
LKKAQMA
LKKAQMA
LKKAQMA
LKKAQMA
275 LKKAQMA
PRKVQVA
VRKVQFA
VRKVQFA
WKKAGLN
280 AKKPSFP

CA 02290722 1999-12-08

PRRATIS
PRRATIS
PRRATIS
PRRATIS
5 PRRATIS
FHRPSEL
FHRPSEL
FHRPSEL
FHRPSEL
10 FHRPSEL
FHRPSEL
IKKANEV
IKKANEV
IKKANEV
15 IKKANEV
FHRPSEL
FHRPSEL
FHRPSEL
IRKVTVS
20 PKRLNFS
YRRVNEL
VKRLSVS
FRGPPGP
MRGSPGP
25 FHLTPDQ
VRLHLLN
VRLHLLN
FRGPPGP
IHPTTGQ
30 IHPTTGQ
VRLPPAA
VHLPNIQ
VHLPNIQ
VRLHLLN
35 FRLSWTA
FRLSWTA
VRPPPAP
VRPPPAP
FRLSWTA
40 VHLSTGA
VHLSTGA
VHPPPVP
VHPPPVP
FHLTPDQ
45 FHLTPDQ
VRLPPAA
IHLSLAP
VHLPNIQ
FRLSWTA
50 FRGLTPP
IRLTLAQ
MRGPPGP
VRGSNGA
IRGPPGP
55 IRGPPGP
VRPLPPA
FRGTLDP
MRLLPLA
IHPTPPA
60 VRGLTGP
MHGLPGP
MRGPPGP
IRGPPGP
IRGPPGP
65 VHLLTLA
FHPTLVA
FHPPPLP
FHPPPLP
FHPPPLP
70 FHPPPLP
FHPPPLP
FHPPPLP
IRGQLGP
MRWLLLY
75 FHPSLGP
IRGPPGP
IRGPPGP
MHGLNAA
VHGLPTP
80 IHLHTPA
FRGPNGA
VHLLPGQ
IRGPPGP
IRGPPGP
85 IRPPPPP
IRGQPGP
IRGQPGP
FRLQTDP
FRLQTDP
90 IRGQPGP
VRLLLGA
VRLLLGA
VRLPTPN
VRLPTPN
95 VRGLTGP
IRGQPGP
VHLSPDA
VHLSPDA
VRGPPGP
100 IRGSTPY
IRGSTPY
IRGSTPY
IRGSTPY
VRPPNGP
105 MRGLLAQ
VRLSPLA
IHGLLPP
FRLSPAA
FHPSLGP
110 FHPSLGP
FRGPNGA
VHPSTVN
VRGLLVN
FRLPPDP
115 FRLPPDP
MRPSNPP
FRGPPLA
FHPPPLP
MRPSNPP
120 MRLLTLA
FRLSPAP
VRGSLGA
VRPPPPP
VRGSNGA
125 VRLPPPA
VRLPPPA
VRLPPPP
VRLPPPP
VHLLLAA
130 MRPPPPA
IRLPTGP
IRWLLAA
IRWLLAA
IRWLLAA
135 IRWLLAA
IRWLLAA
IHPSLLP
VRLPTGY
VRLWNPQ
140 WRGLLVP
VRGPPGP
VRGPPGP
VRPLPPA
VRPLPPA
145 VRPLPPA
IHGPNLY
IHGPNLY
IHGPNLY
WRLTPPA
150 WRLTPPA
FHPHPGP
VRGSLGA
WRGTNVN
WRGTNDQ
155 FRPPPPP
VRPLPPA
VRGWLLA
FHPPPLP
IHLLPGQ
160 WRGHLIA
VHLLPPP
VHLLPPP
VRGSLGA
IRLTPLA
165 VHLLPPP
IHGLNAA
WRLTPPA
VRLWLDN
WRPSLVP
170 VRGTLAY
VRGTLAY
FHLQPVP
FHLQPVP
MRGPTPN
175 FRGSLDY
VRLHPTA
WRGTNVN
VRLHPTA
FRGPLVP
180 VHGLPTP
IRGWPLP
VHLLTLA
IHGLLPP
MRLLTLA
185 MRLLTLA
VRLLLGA
VRLLLGA
VRLLLGA
VRLLLGA
190 VHLSTGA
VHLLTPQ
FRGWNDY
FRGHLDN
VRGSNGA
195 VRGSNGA
VRGTPVP
IRWTPIQ
IRGPPPP
IHGLLPP
200 IHWQPPA
MHGLNAA
MHGLNAA
FRPPPLP
FRLSPAP
205 FRLPPDP
FRLPPDP
FRLPPDP
FRLPPDP
IRGPPGP
210 FRLSPAP
WRGTNVN
VHGSPPA
IRPQLAA
FHLTTTN
215 FHLTTTN
IRWQLPQ
IHPSLLP
FHLTTTN
FHLTTTN
220 FHLTTTN
FHLTTTN
FHLTTTN
FHLTTTN
FHLTTTN
225 FHLTTTN
WRPSLVP
FRLSPTA
VRGLTLP
VHLSNGP
230 VRGLTGP
IHGHPGP
IHGHPGP
IRGHPGP
WHGTNDN
235 IHLLLLQ
VRGPNGA
VRGPNGA
VHLLLAA
WRLTPPA
240 FRGPNGA
FRGPNGA
FRGPNGA
FRGPNGA
FRGPNGA
245 VRGSNGA
VRGSNGA
VRGSNGA
VRGSNGA
VRGSNGA
250 VRGSNGA
MRWLLLY
FRLLPVQ
VRLTPAQ
VRLTPAQ
255 VHLSNGP
VHLSNGP
VHLSNGP
IRPSTLN
WRGPNVN
260 VHLPNIQ
VRLTPAQ
IRGPPPP
MRGPPPP
IRGPPPP
265 IRGPPPP
MRGPPPP
MRWTPAA
MRWTPAA
VRGPPGP
270 VRGLTLP
VRGLTLP
WRPQPPA
IHGLPVP
IRLPTGA
275 IRLPTGA
FRLSPTA
MRPLWVQ
VRLLLGA
VRLLLGA
280 VRLLLGA

CA 02290722 1999-12-08

VRLLLGA	VRLLLGA	IHLTPTQ	FRGWNGQ	VRLLLGA
VRLLLGA	VRLPTPN	IHLTPTQ	VRGPPGP	MRGSPGP
VRLLLGA	VRLPTPN	IHLTPTQ	FHPTLVA	FRPPPPY
VRLLLGA	VRLLLGA	IHLTPTQ	FHPTLVA	FHPTLVA
VRLLLGA	VRLLLGA	IHLTPTQ	VHGPTTP	VRLLNPN
VRLLLGA	VHGPTTP	IHGLPVP	VRLLLGA	FRLLTGQ

Example 5

Suitable phage display libraries including, but not limited to the Ph.D. Phage Display 12-mer peptide library (NEB) were panned against the H11 antibody (including and not limited to the IgM , IgGI, scFv and other antibody fragments) exactly as described in the relevant NEB technical bulletin. See page 11 Sloan Kettering Patent WO 99/22761 for full details. Phage particles were prepared from individual clones and DNA was extracted and sequenced using the Applied Biosystems automatic sequencer and the deduced amino sequences were obtained.

EXAMPLE 6

METHOD OF OBTAINING MAB H11

Mab NBGM1/H11 ("H11"), is a human monoclonal IgM antibody reactive against the following human tumor tissues and corresponding tumor cell lines: glioma, malignant melanoma, colon adenocarcinoma and breast adenocarcinoma. In vitro characterization of Mab NBGM1/H11 is shown in Example 4.

Fusion of H11 was accomplished by fusing 8 x 10^6 peripheral blood lymphocytes obtained from a 64 year old male with a low grade glioma with the TM-H2-SP2 human myeloma cell line. TM-H2-SP2 cell line is the immunoglobulin non-secreting subline of the parental cell line TM-H2, a hypoxanthine guanine phosphoribosyltransferase (EC 2.4.2.8)-deficient derivative of an unknown human myeloma-like line selected in 0.8% methylcellulose for its resistance to 6-thioguanine (6 µg/mL) and failure to grow in hypoxanthine-aminopterin-thymidine medium. The karyotype of TM-H2-SP2 is 46±2, XX.

The resultant viable hybridoma cells were plated (0.2 mL/well) into 40 microwells at a density of 2 x 10^5 cells/mL. The frequency of outgrowth from fusion H11 was 12 of 40 (30%) potential hybridoma-containing wells. Outgrowth resulting from sustained growth is defined as prolonged growth with culture expansion for

periods longer than 3 months; instances of hybridoma growth failure occurring later than 3 months post-fusion were not observed.

Screening of hybridoma clones was performed by antigen-capture enzyme-linked immunosorbent assay (ELISA) in microtiter plates using polyclonal anti-human IgM or IgG as coating antigen. A hybridoma culture supernatant was positive if the measured optical density (O.D.) value exceeded the mean background level of a control culture supernatant by greater than two standard deviations (S.D.).

Selection of a hybridoma clone was performed by cell-fixed ELISA. Culture supernatants from 6 microtiter wells, which tested high for IgM or IgG secretion, were screened against the following previously attached and fixed human tumor cell lines: glioblastoma (SKMG-1 and D-54MG); melanoma (A-375); and colon adenocarcinoma (SK-CO-1). A hybridoma supernatant was considered to be positive if the measured O.D. value exceeded the mean background level of control culture supernatants by greater than two S.D. Mabs produced by hybridoma NBGM1/H11, obtained in this manner, continues to be reactive against these tumor cell lines. The "H11" antibodies are $IgM_{(k)}$.

The methods used for the characterization of Mab NBGM1/H11 include: antigen-capture ELISA, antigen ELISA, cell-fixed ELISA, flow cytometry, immunoperoxidase staining of human tumor cell lines and immunohistochemistry of human tumor and normal tissues (see following examples).

Binding characteristics of this human Mab to human tumor cell lines as determined by flow cytometry, immunoperoxidase staining, cell-fixed ELISA and antigen ELISA (i.e., tumor cell freeze-thaw extracts) are presented below.

EXAMPLE 7

FLOW CYTOMETRIC ANALYSIS OF MAB H11 BINDING TO HUMAN GLIOBLASTOMA (SKMG-1) AND MELANOMA (A-375) CELL LINES

In order to determine H11 binding to intact tumor cells, anchorage-dependent tumor cells growing in T-flasks were detached by incubation with PBS-EDTA and examined by flow cytometry. Cells were collected by low speed centrifugation, washed with ice-cold PBS-1% FBS, centrifuged and the supernatant aspirated. The cell pellet was resuspended in culture medium spiked with one of the following: a control human melanoma IgM; hybridoma NBGM1/H11 culture supernatant; or PBS containing purified Mab H11; and incubated on ice for 30 minutes. After incubation, the cells were collected by centrifugation, washed by resuspension in PBS-FBS and centrifuged. The cell pellet was then incubated for 30 min with FITC-conjugated goat anti-human IgM. After incubation, the cells were washed with PBS-FBS. Finally, the cells were resuspended in PBS-FBS followed by addition of propidium iodide (PI) and washed. PI-positive and FITC-positive cells were analyzed by flow cytometry.

The results of the flow cytometric analyses are shown in Figures 1 and 2. These results indicate that crude and purified forms of Mab H11 bind to a cell surface-associated antigen(s) expressed on glioblastoma (SKMG-1) and melanoma (A-375) live human tumor cell lines.

EXAMPLE 8

ANALYSIS OF MAB H11 BINDING TO FREEZE-THAW EXTRACTS OF HUMAN TUMOR CELL LINES BY ELISA

In order to determine the ability of H11 to bind specifically to human tumor samples, ELISA plates were coated with human tumor cell extracts prepared by repeated freezing and thawing of glioblastoma (SKMG-1), breast adenocarcinoma (BT-20, MB-468 and MB-453), colon adenocarcinoma (SK-CO-1 and HT-29) cells.

The coated ELISA plates were incubated for 16-18 hours at 2-8°C. The plates were blocked with PBS-3% BSA for 1 hr at room temperature. Then the plates were incubated with either biotinylated Mab H11 in PBS or biotinylated control IgM in PBS or culture medium for 2 hrs at room temperature. The plates were washed and incubated with streptavidin-conjugated alkaline phosphatase for 2 hrs. After washing, p-nitrophenyl phosphate substrate was added to each plate and, after incubation, the plates were read at 405 nm in an ELISA plate reader.

The binding of Mab H11 to the tumor cell extracts is shown in Figures 3 and 4. These results indicate that Mab H11 binds to tumor cell extracts prepared from glioblastoma, breast adenocarcinoma and colon adenocarcinoma cells in a dose-dependent manner.

EXAMPLE 9

BINDING OF MAB H11 TO HUMAN TUMOR CELLS DETERMINED BY IMMUNOPEROXIDASE STAINING

In order to determine immunoreactivity of H11, the following experiment was performed. Tumor cells grown in 24-well plates on coverslips for 48-96 hrs. were washed. The cells were washed with PBS, fixed with formaldehyde and incubated with 5% normal goat serum on PBS for 30 min. After washing, the cells were incubated for 2 hrs with either hybridoma NBGM1/H11 culture supernatants or purified Mab H11 in PBS or culture medium spiked with control human myeloma IgM. The cells were then washed and incubated with anti-human IgM conjugated to HRP. Finally, the cells were washed, incubated with DAB substrate to visualize Mab H11 binding, counter-stained with hematoxylin and mounted in GVA.

The results of the immunoreactivity of Mab H11 are shown in Table 1 where reactivity is indicated as negative (--), weak positive (+), positive (++), strong positive (+++). These results indicate that, as determined by immunoperoxidase

staining, the epitope recognized by Mab H11 is expressed by a number of different types of human tumor cell lines.

Table 1

CELL LINES/TYPE OF TUMOR	REACTIVITY	
	Control IgM	Mab H11
HUMAN GLIOBLASTOMA		
SKMG 1	--	+++
U-118 MG	--	++
U-87 MG	--	++
HUMAN MALIGNANT MELANOMA		
A-375	--	+++
SK-MEL-5	--	++
HUMAN COLON ADENOCARCINOMA		
SK-CO-1	--	++
HUMAN BREAST ADENOCARCINOMA		
MG-468	--	++
MB-453	--	+
BT-20	--	++
BT-474	--	++
HUMAN KIDNEY ADENOCARCINOMA		
SW-839	--	++
HUMAN OSTEOGENIC SARCOMA		
SAOS-2	--	++
HUMAN OVARY ADENOCARCINOMA		
SK-OV-3	--	++

EXAMPLE 10

BINDING OF MAB H11 TO HUMAN TUMOR CELL LINES DETERMINED BY CELL-FIXED ELISA

The binding of H11 to human tumor cells and cell lines was also determined by cell-fixed ELISA. Growing tumor cells were detached from the T-flask surface by incubating with EDTA-PBS. Cells were collected by centrifugation, washed with PBS, resuspended in culture medium, counted, and aliquots of 5-10 x 10^6 cells placed in each well of 96-well ELISA plates. After allowing the cells to attach to the plates, the culture supernatants were removed and the plates were blocked with PBS-BSA. The cells were then incubated with different concentrations (1-20 μg/mL) of either Mab H11 or control human myeloma IgM for 2 hrs. After incubation, the plates were washed, incubated with biotin-conjugated goat anti-human IgM, washed again and incubated with streptavidin-conjugated alkaline phosphatase. Finally, the plates were washed, incubated with p-nitrophenyl phosphate substrate and read at 405 nm in an ELISA plate reader.

Results of the reactivity of Mab H11 to human tumor cell lines by cell-fixed ELISA are shown in Table 2 and Figure 5. In Table 2, Control IgM 10μg/mL w ere used for testing the reactivity, and values are given as ± S.D. These results indicate that: 1) Mab H11 reacts strongly with glioblastoma cells (SKMG-1), even at a low concentration of 1 μg/mL, whereas control IgM at 20 μg/mL does not react with SKMG-1 cells; and 2) Mab H11 recognizes the tumor antigen(s) present on numerous tumor cell lines (breast adenocarcinoma, colon adenocarcinoma, malignant melanoma, neuroblastoma, glioblastoma, lung adenocarcinoma, small cell lung carcinoma and prostate adenocarcinoma). The degree for Mab reactivity varies both with the type of cancer and the tumor cell lines.

CA 02290722 1999-12-08

TABLE 2

Cell lines/Tumor Type	Reactivity (O.D. at 405 nm)	
	Control IgM	Mab H11
Human Glioblastoma		
SKMG-1	0.21 ± 0.01	0.95 ± 0.06
D-54-MG	0.13 ± 0.02	0.43 ± 0.07
U-87MG	0.13 ± 0.02	0.60 ± 0.01
Neuroblastoma		
SK-N-SH	0.14 ± 0.02	0.96 ± 0.06
SK-N-MC	0.17 ± 0.03	1.00 ± 0.05
Malignant Melanoma		
A-375	0.25 ± 0.04	1.25 ± 0.04
SK-MEL-5	0.18 ± 0.03	1.42 ± 0.04
SK-MEL-28	0.19 ± 0.03	1.79 ± 0.05
Breast adenocarcinoma		
MB-453	0.68 ± 0.18	2.85 ± 0.14
MB-468	0.60 ± 0.03	2.39 ± 0.10
SK-BR-3	0.60 ± 0.03	2.14 ± 0.13
T47D	0.58 ± 0.01	2.13 ± 0.04
BT-20	0.57 ± 0.02	2.07 ± 0.13
BT-474	0.61 ± 0.03	2.20 ± 0.17
Lung adenocarcinoma		
SW-900	0.20 ± 0.02	0.68 ± 0.10
SK-LU-1	0.19 ± 0.02	0.57 ± 0.07
A-427	0.22 ± 0.01	0.88 ± 0.07
Small cell lung carcinoma		
NCI-H69	0.25 ± 0.04	1.42 ± 0.20
NCI-H82	0.20 ± 0.09	1.16 ± 0.13
Colon adenocarcinoma		
SK-Co-1	0.27 ± 0.03	0.98 ± 0.11
HT-29	0.37 ± 0.02	1.78 ± 0.20
Kidney Adenocarcinoma		
SW-839	0.02 ± 0.01	1.43 ± 0.01
Prostate adenocarcinoma		
PC-3	0.17 ± 0.01	0.60 ± 0.01
DU-145	0.15 ± 0.01	0.52 ± 0.01
Osteogenic Sarcoma		
SAOS-2	0.24 ± 0.02	1.22 ±0.07
U-2 OS	0.13 ±0.04	1.93 ± 0.05

Bladder Cell Carcinoma T-24	0.13 ± 0.01	1.25 ± 0.03
Ovarian Adenocarcinoma SK-OV-3	.012 ± 0.01	1.14 ±0.02
Larynx Carcinoma HEP-2	0.25 ±0.01	1.25 ± 0.01
Normal Human Fibroblast GM-8333	0.13 ± 0.01	0.39 ± 0.01

EXAMPLE 11
IMMUNOANATOMIC DISTRIBUTION AND IMMUNOPATHOLOGIC ANALYSIS OF H11

Immunohistochemistry was used to determine expression of H11 for evaluation of micro-anatomical detail and heterogeneity in tissues and tumors. Limitations of this technique include possible false negative results due to low levels of expression of the molecule under study, as well as false positive results (cross-reactivity) due to antibody-binding to similar epitopes or epitopes shared by other antigens. To address these limitations, this study was carried out at the highest concentration of antibody that did not show non-specific binding by a control antibody. This allowed for detection of all levels of cross-reactivity in different tissues. In addition, fixation analysis to establish the best combination of antigenic staining intensity and morphological preservation, was performed. The present example presents results obtained from IMPATH Inc., New York, which was retained to study the cellular specificity and antigen expression of H11, on a selected panel of cryostat-cut frozen sections of normal and tumor tissues. The study used an indirect immunoperoxidase technique.

Histologically normal human tissues were obtained from surgical and autopsy specimens. These fresh tissues were embedded in OCT compound (Miles Laboratories, Inc., Naperville, IL) in cryomolds and snap-frozen in isopentane,

cooled by liquid nitrogen. These tissues from IMPATH's frozen tissue bank were then cut at 5 microns, placed on poly-L-lysine coated slides, air-dried, and stored in a -70°C tissue bank until needed.

H11, received on wet ice and stored at 2-8°C, was supplied non-biotinylated at a concentration of 200 μg/mL, total volume of 3.0 mL. A human myeloma IgM (Pierce Cat. #31146), also supplied by Novopharm Biotech, Inc., was used as the negative control antibody. Both the negative control antibody and H11 were diluted in phosphate buffered saline (PBS) to the same working concentrations dictated by titration analysis of H11. The peroxidase-labeled secondary antibody was a goat anti-human IgM (American Qualex, San Clemente, CA, lot #A112PN) diluted in PBS to 1:500.

Immunoperoxidase Techniques: Immunohistochemical studies were performed using an indirect immunoperoxidase method. The cryostat cut sections were removed from the -70°C freezer, air-dried and fixed (fixation details provided below). Tissue sections were blocked for 10 minutes with 5% normal goat serum diluted in PBS, then incubated with the primary antibody overnight at 4°C. Slides were washed in PBS, followed by a wash with 0.5% Tween/PBS solution, then another wash in PBS. Endogenous peroxidase activity was blocked with a 30 minute 3% hydrogen peroxide/methanol incubation, followed by 3 washes of PBS. The sections were then incubated with goat anti-human IgM (peroxidase-labeled) secondary antibody for 15 minutes, at room temperature, and washed in PBS as described above.

The peroxidase reaction was visualized by incubating tissue sections for 2–5 minutes with 3, 3-diaminobenzidine-tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO). Tissue sections were thoroughly washed, counterstained with a modified Harris hematoxylin (Fisher Scientific, Fairlawn, NJ) dehydrated through graded alcohols, cleared in xylene, and coverslipped. Tissues that demonstrated high

levels of background staining with the negative control antibody were stained again utilizing more extensive washing.

Human breast carcinoma (F95-036), supplied by IMPATH, was used as the positive control for H11. Negative controls substituted the primary test antibody with purified human myeloma IgM.

The purpose of the fixation analysis was to establish the conditions which provide the optimal combination of antigenic staining intensity and morphologic preservation. The positive control tissue was tested with five fixation protocols, including no fixation. The fixation protocols tested were 10% neutral buffered formalin (23-25°C), acetone (2-8°C), methyl/acetone (1:1 V/V, 2-8°C) and 95% ethanol (23-25°C). For this study, 10% neutral buffered formalin (NBF) gave optimal results for H11.

Using 10% NBF as the fixative, serial antibody dilutions (20.0 µg/mL to 0.1 µg/ml) were tested on the positive control, human breast carcinoma. A concentration of 10.0 µg/mL of antibody H11 gave optimal results—maximum staining intensity without significant background staining of the negative control.

The results obtained are depicted in Tables 3 and 4. Table 3 depicts H11 reactivity on normal tissues and Table 4 shows H11 reactivity on human tumors.

TABLE 3

Tissue	Tested Positive/Total	Range of Reactivity (0-3+)
Adrenal	0/3	0
Bladder	0/3	0
Bone Marrow	1/3	1+
Brain	0/3	0
Breast	0/3	0
Cervix	0/3	0
Esophagus	0/3	0
Eye	0/3	0
Heart	0/3	0
Kidney	0/3	0
Large Intestine	0/3	0
Liver	0/3	0
Lung	0/3	0
Lymph Node	0/3	0
Muscle	0/3	0
Ovary	0/2	0
Pancreas	0/3	0
Parotid	0/3	0
Pituitary	0/1	0
Prostate	0/3	0
Skin	0/3	0
Small intestine	0/3	0
Spinal cord	0/3	0
Spleen	0/3	0
Stomach	0/3	0
Testis	0/3	0
Thymus	0/3	0
Thyroid	0/3	0
Tonsil		1+
Uterus	0/3	0
White Blood Cell	0/3	0

TABLE 4

Tumor	Tested Positive/Total	% of Tumor Cells Staining	Range of Reactivity (0-3+)
Breast carcinoma		30-90	1-3+
Colon carcinoma	3/3	40-70	1-2+
Glioma	4/6	30-90	1-2+
Gastric carcinoma	3/3	30-50	1-2+
Lung adenocarcinoma	¾	10-70	1-2+
Lung squamous carcinoma	3/3	10-95	1-3+
Lung small cell carcinoma	½	30	1+
Lymphoma	8/8	10-95	1-3+
Melanoma	3/3	20-95	1-2+
Ovarian carcinoma	3/3	20-30	1-3+
Prostate carcinoma	3/3	20-95	1-2+
Sarcoma	0/3	0	0

The results obtained indicate that weak (1+) to strong (3+) reactivity was observed in over 70% of the positive control sample. The antigen recognized by H11 has a restricted pattern of distribution. H11 was largely unreactive with normal human tissues tested in the IMPATH system. All simple epithelial cells, as well as the stratified epithelia and squamous epithelia of different organs, were found to be unreactive. No reactivity was observed in neuroectodermal cells, including those in the brain, spinal cord and peripheral nerves. Mesenchymal elements such as skeletal and smooth muscle cells, fibroblasts, and endothelial cells were negative. Tissues of lymphoid origin including bone marrow, lymph node, spleen, and thymus were largely unreactive with antibody H11. Weak (1+) reactivity was observed in rare cells in one specimen of bone marrow and in the germinal centers of one of three specimens of tonsil tested.

Positive immunoreactivity was observed in almost all specimens of tumor tested including breast, colon, glioma, gastric, lung (adeno, squamous, and small cell), lymphoma, melanoma, ovarian, and prostate. Reactivity was seen in 10% to greater than 95% of the tumor cells present in these specimens; staining intensity

ranged from weak (1+) to strong (3+). Antibody H11 was, however, unreactive with all three specimens of sarcoma tested. Some, but not all, normal counterparts of the tumor cells, when present in the specimens, were reactive with H11. A few normal cells present in breast, gastric and prostate carcinoma were reactive with antibody H11. The large granular cells that were reactive with antibody H11 are believed to be inflammatory cells of the eosinophil-mast cell lineage.

In summary, antibody H11 is largely unreactive with normal human tissues with the exception of some normal cells such as infiltrating leukocytes, tissue present in tumors. The H11 antibody detects an antigen that is expressed in almost all of the tumors tested in the present study.

EXAMPLE 12

PURIFICATION OF SPPCS

(a) Purification of mixtures.

Purification of SPPC mixtures using ADP-affinity chromatography is described in Peng et al. (1997) *J. Immunol. Met.* 204 13-21; and WO98/12208. In particular, a semi-purified cell extract is added to a column containing an ADP matrix and a buffer containing ADP is then added to the column to elute the SPPCs. Generally, a tumor cell extract can be prepared by standard techniques in the art, with specific attention paid to inhibiting protease activity, preferably by freeze thaw extract methodology as generally described in Chen et al (1994) *J. Immunol.* 152:3-11. Preferably the protease activity is inhibited using PMSF and aprotinin.

(b) Purification of SPPC containing hsp70.

C-antigen was isolated in the following manner. A-375 cells (human melanoma cell line) were grown in tissue culture to a cell density of 50-80% confluent, disrupted, and an extract made by freeze-thaw. In detail, after cell harvest, cells were centrifuged at 1500 rpm for 10 min. The cells were washed twice in a PBS/1 mM PMSF/10μg/mL aprotinin solution. After washing, the pellet was resuspended in the wash solution and the cell concentration was adjusted to 10-20 x

10^6 cells/mL. This suspension was then subjected to five freeze-thaw sequences consisting of freezing in a dry-ice-acetone solution, followed immediately by thawing in a 37°C water bath. After the freeze-thaw treatments the extract mixture was centrifuged at 4°C, 2500rpm for 30 minutes.

The resulting supernatant was combined with 3M ammonium sulfate buffer in a 2:1 ratio. This sample was then loaded onto a general purpose hydrophobic chromatographic media (preferably Phenyl Sepharose) at a rate of 0.5 mL/min using a pump. The column was connected to an FPLC system. Once loaded, the column was washed with 15 column volumes (CV) of Buffer A (50 mM sodium phosphate/1M ammonium sulfate pH 7.0). The bound proteins were eluted step-wise with buffer B (50 mM sodium phosphate pH 7.0). Active fractions were determined by immunological methods. During elution, the bulk of the bound proteins were eluted with 30% Buffer A /70% buffer B. The 70% buffer B elution was followed by 100% buffer B. SPPC was eluted in this latter fraction. This positive fraction was concentrated on a membrane concentrator with a MW cut-off of 10 kD (preferably a Centriprep 10). The concentrated sample was passed through a buffer exchange media (preferably G-25) to the ADP-agarose chromatographic Buffer A (20 mM Tris-acetate, 20 mM NaCl, 3 mM $MgCl_2$, pH 7.5).

Six mL of the buffer exchanged material was incubated overnight with an additional 4 mL Buffer A and 5 mL ADP-agarose at 4°C on a platform shaker. Following incubation, the mixture was poured into a XK16 x 40 column. The column was washed with the ADP-agarose chromatographic Buffer A until the OD at 280 reached base-line. The column was further washed with 0.5M NaCl in chromatographic-Buffer A and re-equilibrated with Buffer A. The bound protein was then eluted with 3 mM ADP in the ADP Buffer A and fractions collected. The active fraction was concentrated on a membrane concentrator with a MW cut-off of 10 kD (preferably Amicon).

The concentrated, eluted, sample was diluted with anionic chromatographic Buffer A (20 mM Tris pH 7.8) at a 1:10 dilution. One mL of diluted sample was loaded onto a strong anionic column (preferably a Mono Q Sepharose) attached to an FPLC. The flow rate was set at 1 ml/min. Fractions were collected and the antigenic fraction identified as outlined above. This three-step procedure gives a reasonable homogeneous active C-antigen (>95%).

Subsequently, after final concentration from the anionic column, 15µl of 95% purified SPPC was mixed 50/50 with 2X Laemmli's buffer. The sample was run under native, non-denaturing, conditions (no SDS, mercaptoethanol or boiling). After completion of electrophoresis, the separated protein were blotted onto a membrane (PVDF or nitrocellulose) again under non-denaturing conditions. Identification of the antigen location on the blotted membrane was confirmed by incubation with H11-IgG followed by an appropriately labeled secondary antibody. The C-antigen can then be "cut" and eluted from the membrane and subjected to further analyses.

An alternate method for the purification of SPPCs was developed from the creation of affinity chromatographic media of C-antigen-specific IgG antibodies or fragments thereof, in the present case using H11 IgG described in WO97/44461. A 5 mL sample from a hydrophobic column (preferably Phenyl Sepharose) was incubated with 2 mL of H11 Sepharose. The IgG-Sepharose/sample was incubated over-night at 4°C on a rotary shaker. After incubation the mixture was poured into a small chromatographic column (preferably BioRad 10 ml Econo-Column). The column was washed with ten CV of PBS (pH 7.4) followed by three CV of 0.5 M NaCl in PBS. The affinity column was then re-equilibrated with PBS. Following equilibration, C-antigen was eluted using a glycine buffer pH 2.8. The eluted material is concentrated on a micro-pore concentrator (preferably Centriprep 3). The acid elution results in the dissociation of SP from its peptide. The small molecular weight fraction (peptide) was concentrated with a peptide concentrator (preferably Microcon SCX). The purified SP was retained on the micro-pore concentrator.

EXAMPLE 13

THERAPEUTIC EFFECT OF H11 SCFV ON HUMAN TUMOR XENOGRAFTS

Tumor growth inhibition by H11 scFv in a xenograft mouse model.

The potential of H11 scFv as a cancer therapeutic agent was explored using a human tumor xenograft mouse model (Balb/c athymic nude mice). In these studies, outlined below, an anti-tumor effect was found to be associated with H11 scFv treatment in mice implanted with one of the following human tumors: non-Hodgkin's B-cell lymphoma, prostate adenocarcinoma, breast adenocarcinoma, and melanoma. The anti-tumor effects observed, at the doses given, include reduced tumor size, tumor regression, reduced metastatic index and increased survival.

A. Non-Hodgkin's Lymphoma

Mice that were implanted with human non-Hodgkin's lymphoma (Daudi) tissue exhibited a smaller mean tumor volume than their control counterparts (n=3) after being treated with H11 scFv (n=9) and H11 scFv-restrictocin (n=9). The total dose of H11 scFv given was 0.5 mg/kg in a regimen that consisted of 0.1 mg/injection given intravenously (i.v.), 5 times, once, every 4 days. In the H11 scFv-restrictocin-treated group, 2 of 8 animals remaining on Day 38 of the study exhibited partial tumor regression. Despite the reduction in mean tumor volume, the differences found on the last day of the study (Day 38) were not significantly different from that determined for control animals. (p=0.405, Student's t-test, H11 scFv; p=0.423, H11 scFv-restrictocin).

In a follow-up to the first lymphoma study, mice implanted with Daudi were treated with a total dose of 1 mg/kg of H11 scFv (n=13). These H11 scFv-treated mice demonstrated a statistically significant suppression of tumor growth compared to controls at Day 42 (n=8), (P=0.004, Student's t-test). Moreover, 31% (4/18) of the H11 scFv-treated animals exhibited tumor regression, with 3 being partial and 1 being complete. No spontaneous regression was observed in the control animals.

The dose used was administered at 0.1 mg/kg i.v., given once a day for 5 days, rested for 9 days, and then retreated for 5 days as before.

A third study implanted Balb/C athymic mice with varying sizes (4-10, 30-60 and 100-200 mm^3) of human non-Hodgkin's B-cell lymphoma tumor tissue and given a total dose of 20 mg/kg H11 scFv. The dose regimen included 4 cycles of H11 scFv treatment (1 cycle of treatment constituted 5 daily i.v. injections of 1 mg/kg with each cycle being separated by 2 days of rest). There were 22 animals in each treatment group. At the end of treatment all mice treated with H11 scFv showed a reduction in tumor volume compared to controls. The difference was statically significant at Day 51, compared to controls, for the 4-10 and 30-60 mm^3 size tumors ($p=0.02$ and $p=0.006$, respectively).

Tumor regression was seen in 22% (4/18), 10.5% (2/19) and 12% (3/25) of the mice having tumor sizes of 4-10, 30-60 and 100-200 mm^3, respectively, at the onset of treatment. Most notably, for 75% (3/4) of the mice with a tumor size of 4-10 mm^3 at the beginning of the H11 scFv treatment, tumor regression was complete. Control animals did not show tumor regression.

B. Melanoma

Mice were implanted with a human melanoma tumor (GI-105) and treated with a total of 1 mg/kg of H11 scFv (0.1 mg/kg) once a day for 5 days, rested for 9 days, and then retreated for 5 days. Although the mean tumor volumes of the H11 scFv-treated and control groups at the conclusion of the study (Day 42) were not statistically different, the survival rate was higher in the H11 scFv-treated group. The animals treated with drug had a mortality rate of 21% (3/14). In contrast, 50% (4 of 8) of the control mice died.

C. Breast Adenocarcinoma

A trial involving mice implanted with a highly metastatic human breast adenocarcinoma (GI-101) was also conducted. These animals were given 5 daily i.v.

treatments (0.1 mg/kg) of H11 scFv, rested for 9 days, retreated for 5 days and then given twice weekly injections at the same dosage for approximately 7 weeks. The total dose given was 2.4 mg/kg. All animals were sacrificed on day 77 and the lungs removed for histologic examination and quantification of metastatic foci. The number of metastatic foci was expressed as a metastatic index using the following procedure. Briefly, on each slide, two different lung sections were measured with calipers and the number of metastatic foci in each section was counted. Each focus was counted as containing 1-10 cells, 11-50 cells or greater than 50 cells. When the metastatic index was calculated, foci with 1-10 cells were assigned a value of 1, foci containing 11-50 cells were assigned a value of 5 and foci containing greater than 50 cells were assigned a value of 10. The number of foci of each type was multiplied by it's assigned value and these numbers were added together to obtain the total metastatic index (MI). The MI was then divided by the number of mm^2 of lung screened. A final score of MI/mm^2 was then reported.

Although the mean tumor volume of the H11 scFv-treated group was approximately half that calculated for the control group at the end of the study, 237 mm^3 versus 429 mm^3, respectively, the difference was not, statistically different ($p=0.358$ at Day 42). However, the H11 scFv-treated mice exhibited a significantly reduced number of metastatic foci in the lungs than control mice, 14 versus 21, respectively (Chi-square analysis, $p<0.05$).

D. Prostate Cancer

Mice were implanted with a human prostate cancer tumor and then given 4 cycles of H11 scFv treatment (1 cycle of treatment constituted 5 daily i.v. injections of 1 mg/kg with each cycle being separated by 2 days of rest). Upon completion of the i.v. protocol, the same treatment schedule and dose regimen were repeated except that H11 scFv was administered intraperitoneally. The total dose was 20 mg/kg. The treated animals showed marked suppression of tumor size compared to controls. Treatment also had an effect on survival. Thirty-eight percent (6/16) of the mice with

4-20 mm^3 size tumors at the start of treatment with H11 scFv demonstrated long term survival (>100 days). This was significantly different from controls where all animals (10/10) were dead before day 100. Together, these results demonstrate that H11 scFv, when administered to athymic mice bearing human tumor tissue implants, possesses potent *in vivo* anti-cancer activity against human tumors of various origins.

EXAMPLE 14

PREPARATION OF cDNA ENCODING ANTIBODY FRAGMENTS

RNA (mRNA) is prepared from PBL (peripheral blood lymphocytes) of normal or immunized humans using standard methods. cDNA is prepared from the mRNA and used as template for PCR amplification of VH and VL genes. The VH and VL genes are spliced together with a linker region using PCR to create scFv repertoires. Restriction endonuclease sites are added to the gene repertoires to permit forced cloning into a phage display vector.

Gel purified scFv repertoires are digested with the specific restriction enzymes and ligated directly into phage display vector. The resulting recombinant vector is used to transform electrocompetent *E. coli* resulting in the production of a phage antibody library. Phage are processed and incubated with the immobilized antigen, non-binding phage then are removed by washing. Bound phage are eluted with the addition of alkali. Bound phage are used to infect *E. coli* to produce more phage for the next round of selection.

After 3-4 rounds of selection, clones are analyzed for antigen-binding by ELISA. DNA sequencing can be used to characterize unique, high affinity binders. These binders include scFv and a wide variety of other antibody fragments.

EXAMPLE 15

HUMAN MABS DIRECTED AGAINST TUMOR-ASSOCIATED SPPC

Human-human hybridomas secreting monoclonal antibodies (Mabs) specific for tumor-associated SPPCs are generated by fusing peripheral blood lymphocytes (PBL) from a patient presenting with a malignancy. The fusion protocol has been previously described by Galfre and Milstein (1981) *Met. Enzymol*. 73:1-46.

Briefly, PBL isolated by Ficoll gradient density centrifugation are mixed at a PBL:fusion partner ratio of 3:1 in serum-free medium. The fusion partner is an Epstein-Barr nuclear antigen-negative, human myeloma-like cell line, TM-H2-SP2 (Sullivan et al. Hybridoma Technology, pp. 63-68, L. Russ, D. Carlton, eds. Ortho Pharmaceuticals Canada Ltd., Toronto, 1982). The cell mixture is centrifuged (400 xg, 5 min), the supernatant removed and membrane fusion facilitated by the addition of 1 mL of pre-warmed (37°C) 50% polyethylene glycol (PEG) in serum-free medium directly into the pellet over a period of 1 min. One mL of serum-free medium is added again directly into the pellet over 1 min and this step repeated twice more.

An additional 7 mL serum free medium is added slowly (over the course of 2 min) to the pellet with stirring and a final 12 to 13 mL added dropwise to the mixture. After which, the cell mixture is centrifuged (400 xg, 5 min), the supernatant discarded and the cell pellet resuspended to a final cell concentration of 1.0×10^6 cells/mL in complete medium containing hypoxanthine (H, 100 µM) and thymidine (T, 16 µM). A 200 µL volume of the cell mixture is aliquoted into each well of a sterile 96 well flat-bottom tissue culture plate. The following day 100 µL is removed from each well and replaced with 100 µL of complete medium containing aminopterin (A, 2X, 0.8 µM). Several wells containing the fusion partner alone are added to one of the plates to ensure the selectivity of the medium. Every 3 to 4 days, half of the medium is removed and replaced with medium containing HAT and monitored for the growth of hybridomas. Wells containing hybrids are screened for anti-SPPC reactivity using the dot-blot procedure detailed in Example 3. Wells exhibiting reactivity towards the SPPC fraction are expanded into 24 well plates. Supernatants from these cultures are tested against the cell membrane fraction containing the SPPC in the presence and absence of ATP. Clones demonstrating antibody reactivity in the absence of ATP, but negative in the presence of ATP, are cloned by limiting dilution

into 96 well plates at 1 cell per well. All subclones are re-tested and the cloning procedure repeated twice more with the best positive subclones.

Example 16

Identification of peptides containing the consensus peptide motif $X_m(HyX)_3X_m$

Synthesis and screening of an oligonucleotide array is carried out using commercially available DNA GeneChip technology, produced by Affymetrix®. (See US 5,510,270; US 5,744,305; 5,571,639; and US 5,527,681). An oligonucleotide probe array of all DNA sequences corresponding to the consensus sequence HyXHyXHyX, where Hy is valine, leucine or isoleucine, and where X can be any amino acid, with the exception of glycine and proline, is generated. Alternative sets of probes can use less hydrophobic amino acid residues for Hy.

Photolithographic masks designed by computational algorithms are used in manufacturing the probe arrays. (See US 5,571,639). A total of 327,082,769 oligonucleotides are calculated, where Hy can be encoded by any of GUU, GUC, GUA, GUG, UUA, UUG, CUU, CUC, CUA, CUG, AUU, AUC, or AUA and X can be encoded by any of UUU, UUC, UUA, UUG, CUU, CUC, CUA, CUG, AUU, AUC, AUA, AUG, GUU, GUC, GUA, GUG, UCU, UCC, UCA, UCG, ACU, ACC, ACA, ACG, GCU, GCC, GCA, GCG, UAU, UAC, CAU, CAC, CAA, CAG, AAU, AAC, AAA, AAG, GAU, GAC, GAA, GAG, UGU, UGC, UGG, CGU, CGC, CGA, CGG, AGU, AGC, AGA or AGG. Thus, $[13^3 x 53^3]$ results in 327,082,769 possible permutations factored into synthesis of the oligonucleotide array. Approximately 4 GeneChips, each holding up to $1X10^8$ oligonucleotides (See US 5,510,270, column 15, line 32), are hybridized to a cDNA library generated from a tumor cell, for instance the A-375 melanoma cell line. Hybridization data are collected to identify each probe sequence that hybridizes to individual cDNA consensus sequences. The sequence of each probe giving rise to a positive match is recorded and grouped according to the peptide sequence encoded. This subset of probes is referred to as the optimized oligonucleotide array. There can be a maximum number of $[3^3 x 18^3]$ or

CA 02290722 1999-12-08

157,464 possible peptide permutations. Some number of the possible peptide permutations do not exist in the A-375 melanoma cell line. Screening over GeneChips reduces this number to the actual number of peptide consensus sequences that exist in the A-375 melanoma cell line.

Secondary screening over GeneChips is used to identify a consensus sequence motif $X_m(HyX)_3X_m$, where (HyX) consists of the optimized oligonucleotide array identified above and where X is any amino acid, with the exception of glycine and proline, and II $x_m \leq 5$ amino acids.

Secondary screening over gene chips can also be carried out where X_m in the consensus sequence motif $X_m(HyX)_3X_m$ is ascertained by isolating and sequencing cDNAs that hybridize to the optimized oligonucleotide array identified above.

Secondary screening over gene chips can also be carried out where X_m in the consensus sequence motif $X_m(HyX)_3X_m$ is ascertained by isolating and sequencing cDNAs that encode antigenic peptides recognized by H11.

All references cited herein, both supra and infra, are hereby incorporated herein. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

1. A composition comprising at least one SPPC which is specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer.

2. The composition according to claim 1, wherein at least one SPPC contains a non-covalently bound peptide which confers the specific immunogenicity.

3. A composition comprising a plurality of SPPCs, which are specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer.

4. The composition according to claim 3, wherein the SPPCs contain different non-covalently bound peptides, which confer the specific immunogenicity.

5. The composition according to claim 3, where the plurality is selected from the group consisting of, 2-10 SPPCs.

6. The composition according to claim 1, wherein the cancer is selected from the group consisting of astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary

adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bileduct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas.

7. The composition according to claim 1, where the SPPC is specifically immunogenically cross-reactive with more than one type of cancer cell population.

8. The composition according to claim 1, 2, 3 or 4, wherein the SP is a member of a heat shock protein family selected from the group consisting of hsp20-30, hsp60, hsp70, hsp90 and combinations thereof.

9. The composition according to claim 8, wherein the heat shock protein is hsp72.

10. The composition according to claim 8, wherein the heat shock protein is hsp85.

11. The composition according to claim 8, wherein the heat shock protein is hsp96 (gp96).

12. The composition according to claim 8, wherein the peptide is derived from a tumor.

13. The composition according to claim 8, wherein the tumor is obtained from a human or a non-human mammal.

14. The composition according to claim 8, wherein the tumor is human and is selected from the group consisting of astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bileduct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas.

15. A composition of matter comprising an isolated antigen-binding fragment of an antibody which binds specifically to at least one SPPC which is specifically

immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer.

16. The composition of matter according to claim 15, wherein the at least one SPPC contains a non-covalently bound peptide which confers the specific immunogenicity.

17. A composition comprising an antigen-binding fragment of an antibody which binds specifically to a plurality of SPPCs which is specifically immunogenically cross-reactive with one or more cell surface-associated SPPCs specific to a target cancer.

18. The composition according to claim 17, wherein the SPPCs contain different non-covalently bound peptides, which confer the specific immunogenicity.

19. The composition according to claim 15, 16, 17, or 18, wherein the antigen-binding fragment is selected from the group consisting of whole antibodies, bispecific antibodies, chimeric antibodies, Fab, F(ab')2, single chain V region fragments (scFv) and fusion polypeptides.

20. The composition according to claim 19, wherein the antigen-binding fragment is of human origin.

21. The composition according to claim 20, wherein the antigen-binding-fragment is encoded by a phage display library.

22. The composition according to claim 19, wherein the antigen-binding fragment consists essentially of a scFv.

23. The composition according to claim 19, wherein the whole antibody is a human immunoglobulin of any isotype.

24. The composition according to claim 19, wherein the antigen-binding fragment comprises a variable region derived from an IgM and a constant region derived from an IgG.

25. The composition according to claim 19, wherein the fusion peptide comprises the antigen-binding fragment fused to a chemically functional moiety.

26. The composition according to claim 25, wherein the moiety is selected from the group consisting of signal peptides, agents that enhance immunologic reactivity, agents that facilitate coupling to a solid support, bioresponse modifiers, immunotoxins, toxins, detectable labels, paramagnetic labels and drugs.

27. The composition according to claim 26, wherein the agent that facilitates coupling to a solid support is selected from the group consisting of biotin and avidin.

28. The composition according to claim 26, wherein the immunogen carrier is selected from the group consisting of any physiologically acceptable buffer.

29. The composition according to claim 26, wherein the bioresponse modifier is a cytokine.

30. The composition according to claim 26, wherein the cytokine is selected from the group consisting of tumor necrosis factor, interleukin-2, interleukin-4, interleukin-12, granulocyte macrophage colony stimulating factor and γ-interferons.

31. The composition according to claim 26, wherein the drug is an antineoplastic agent selected from the group consisting of radioisotopes, vinca alkaloids, adriamycin, bleomycin sulfate, carboplatin, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, duanorubicin hydrochloride, doxorubicin hydrochloride, etoposide, fluorouracil, lomustine, mechloroethamine

hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mototane, pentostatin, pipobroman, procarbaze hydrochloride, streptozotocin, taxol, thioguanine and uracil mustard.

32. The composition according to claim 31, wherein the vinca alkaloid is selected from the group consisting of vinblastine sulfate, vincristine sulfate and vindesine sulfate.

33. The composition according to claim 26, wherein the toxin is selected from the group consisting of ricin, radionuclides, pokeweed antiviral protein, Pseudomonas exotoxin A, diphtheria toxin, ricin A chain, fungal ribosome inactivating proteins and phospholipase enzymes.

34. The composition according to claim 33, wherein the detectable label is selected from the group consisting of radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, bioluminescent compounds, enzymes, substrates, cofactors and inhibitors.

35. A composition of matter according to claim 1, 2, 3, 4, 16, 17, 18 or 19 wherein the SPPC is specifically immunogenic to the target cancer in a plurality of genetically different individuals having spontaneously arising tumors of the same type.

36. A composition of matter according to claim 1, 2, 3, 4, 16, 17, 18 or 19 wherein the SPPCs are specifically immunogenic with respect to a plurality of different target cancers.

37. A composition comprising a population of one or more different SPPCs predominantly consisting of at least one immunogenic cancer cell surface-

associated SPPC of a target cancer wherein the SPPC comprises a SP associated peptide that renders the SPPC immunogenic with respect to the target cancer.

38. The composition of matter according to claim 16, 17, 18 or 19 further comprising a physiologically acceptable excipient.

39. The composition according to claim 38, wherein the antigen-binding fragment is present in an amount effective to elicit a cancer-associated immune response in a subject upon administration to the subject.

40. The composition according to claim 38, wherein the antigen-binding fragment is selected from the group consisting of whole antibodies, bispecific antibodies, chimeric antibodies, Fab, F(ab')2, single chain V region fragments (scFv) and fusion polypeptides.

41. The composition according to claim 40, wherein the antigen-binding fragment is of human origin.

42. The composition according to claim 41, wherein the antigen-binding fragment is encoded by a phage display library.

43. The composition according to claim 40, wherein the antigen-binding fragment consists essentially of a scFv.

44. The composition according to claim 40, wherein the whole antibody is a human immunoglobulin of any isotype.

45. The composition according to claim 40, wherein antigen-binding fragment comprises a variable region derived from an IgM and a constant region derived from an IgG.

46. The composition according to claim 40, wherein the fusion peptide comprises the antigen-binding fragment fused to a chemically functional moiety.

47. The composition according to claim 44, wherein the moiety is selected from the group consisting of signal peptides, agents that enhance immunologic reactivity, agents that facilitate coupling to a solid support, bioresponse modifiers, immunotoxins, toxins, detectable labels, paramagnetic labels and drugs.

48. The method according to claim 47, wherein the agent that facilitates coupling to a solid support is selected from the group consisting of biotin and avidin.

49. The composition according to claim 47, wherein the bioresponse modifier is a cytokine.

50. The composition according to claim 49, wherein the cytokine is selected from the group consisting of tumor necrosis factor, interleukin-2, interleukin-4, interleukin-12, granulocyte macrophage colony stimulating factor and γ-interferons.

51. The composition according to claim 47, wherein the drug is an antineoplastic agent selected from the group consisting of radioisotopes, vinca alkaloids, adriamycin, bleomycin sulfate, carboplatin, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, duanorubicin hydrochloride, doxorubicin hydrochloride, etoposide, fluorouracil, lomustine, mechlorocthamine hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mototane, pentostatin, pipobroman, procarbaze hydrochloride, streptozotocin, taxol, thioguanine and uracil mustard.

52. The composition according to claim 51, wherein the vinca alkaloid is selected from the group consisting of vinblastine sulfate, vincristine sulfate and vindesine sulfate.

53. The composition according to claim 47, wherein the toxin is selected from the group consisting of ricin, radionuclides, pokeweed antiviral protein, Pseudomonas exotoxin A, diphtheria toxin, ricin A chain, as fungal ribosome inactivating proteins and phospholipase enzymes.

54. The composition according to claim 47, wherein the detectable label is selected from the group consisting of radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, bioluminescent compounds, enzymes, substrates, cofactors and inhibitors.

55. The composition according to claim 38, wherein the stress protein is selected from a heat shock protein family selected from the group consisting of hsp26, hsp60, hsp70, hsp90 and combinations thereof.

56. The composition according to claim 55, wherein the heat shock protein is hsp72.

57. The composition according to claim 55, wherein the heat shock protein is hsp85.

58. The composition according to claim 55, wherein the heat shock protein is hsp96.

59. The composition according to claim 38, wherein the peptide is derived from a tumor.

60. The composition according to claim 59, wherein the tumor is obtained from a human or a non-human mammal.

61. The composition according to claim 60, wherein the tumor is human and is selected from the group consisting of astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bileduct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas.

62. A method of treating a cancer subject comprising administering to the subject an amount of a composition of matter according to claim 38.

63. The method according to claim 62, wherein the peptide is pan-carcinomic.

64. A method of identifying antigen-binding fragments of an antibody specific for a tumor-associated SPPC comprising the steps of:
    (a) generating a suitable phage display library;
    (b) generating stress protein-peptide complex from a tumor;
    (c) screening the product of step (a) with the product of step (b) to obtain phage which display an antigen-binding-fragment that binds specifically to SPPC; and,
    (d) screening the phage obtained in step (c) for cell surface tumor-associated reactivity.

65. A method of isolating an antigenic tumor-associated SPPC, comprising the steps of:
    (a) fractionating a tumor-cell extract on an antigen-binding fragment affinity medium to bind the complex;
    (b) applying the eluate from the affinity medium to molecular sieve which is capable of separating the stress protein from the peptide;
    (c) isolating the peptide; and
    (d) re-associating the stress protein with the isolated peptide.

66. A method as claimed in claim 65, wherein said stress protein is a member of the HSP70 family.

67. A method as claimed in claim 66, wherein said stress protein is HSP72.

68. A method as claimed in claim 64, wherein said SPPC is C-antigen.

69. A method as claimed in claim 64 wherein said SPPC is first fractionated on a hydrophobic column to isolate a hydrophobic fraction.

Fig. 1

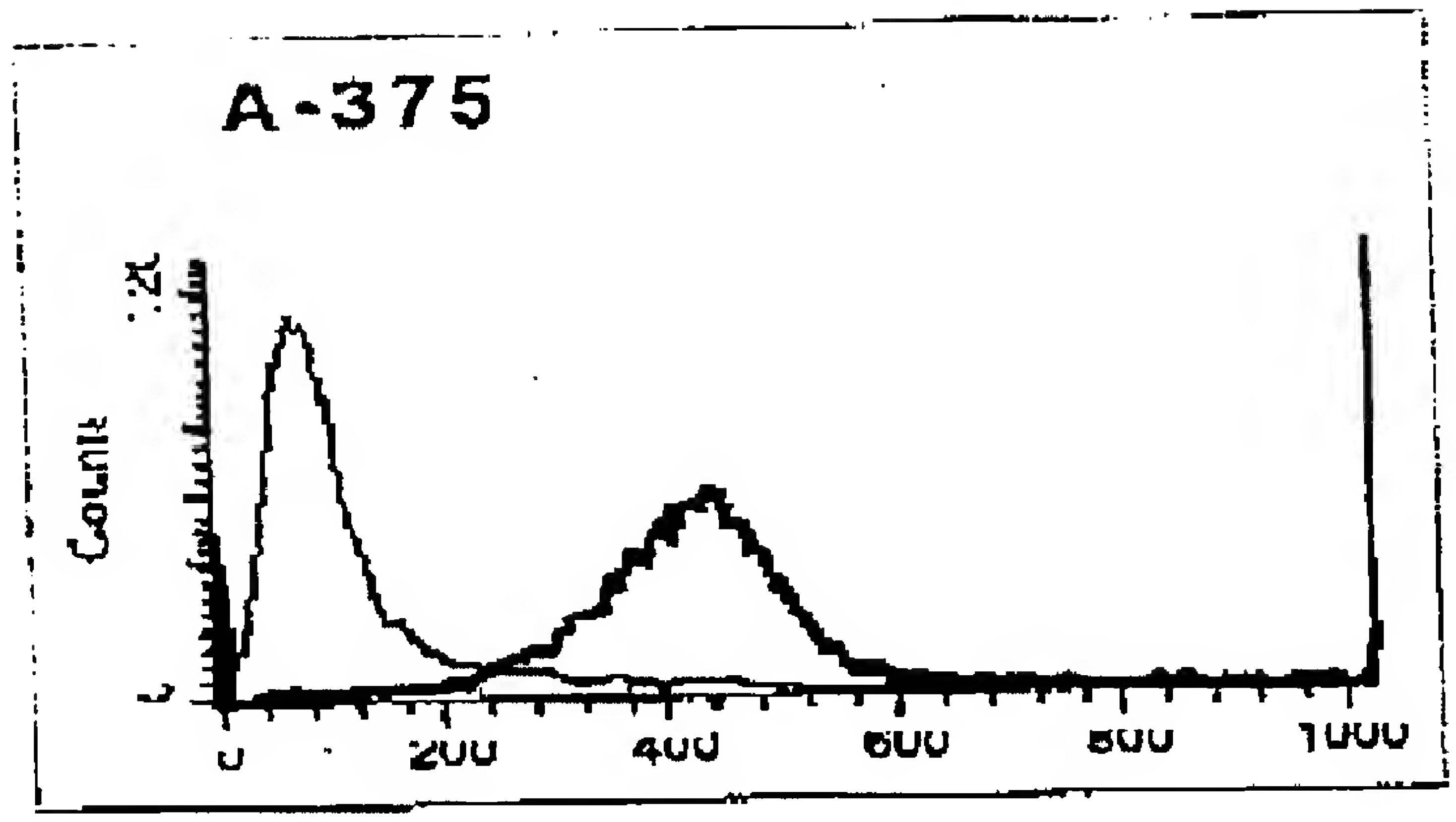

A-375
Counts
0
200
400
600
800
1000

Fig. 2

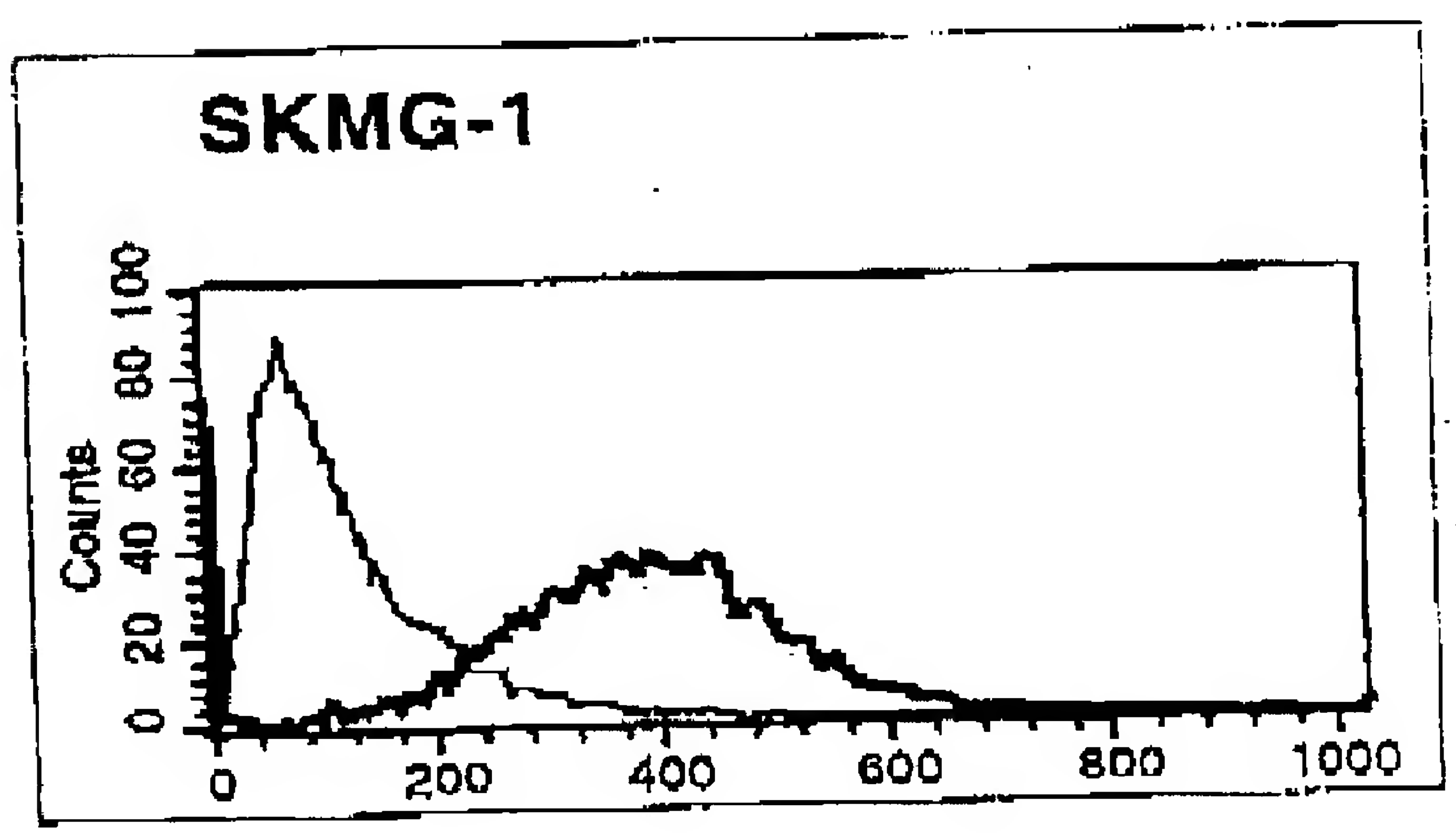

SKMG-1
Counts
0
20
40
60
80
100
0
200
400
600
800
1000

Fig. 3

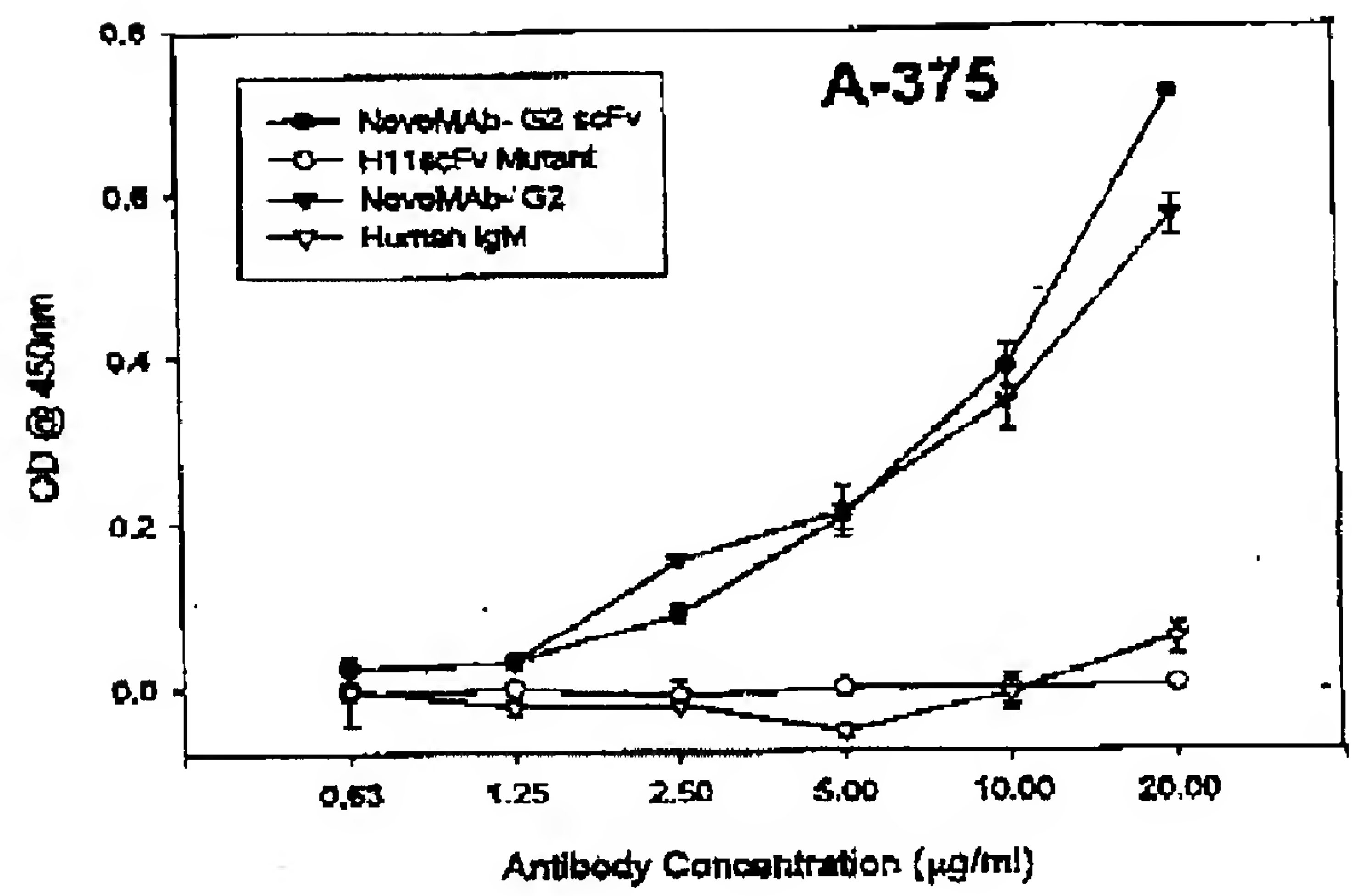

A-375
NovoMAb- G2 scFv
H11scFv Mutant
NovoMAb- G2
Human IgM
OD @ 450nm
0,8
0,6
0,4
0,2
0,0
0,63
1.25
2.50
5.00
10.00
20.00
Antibody Concentration (µg/ml)

Fig. 4

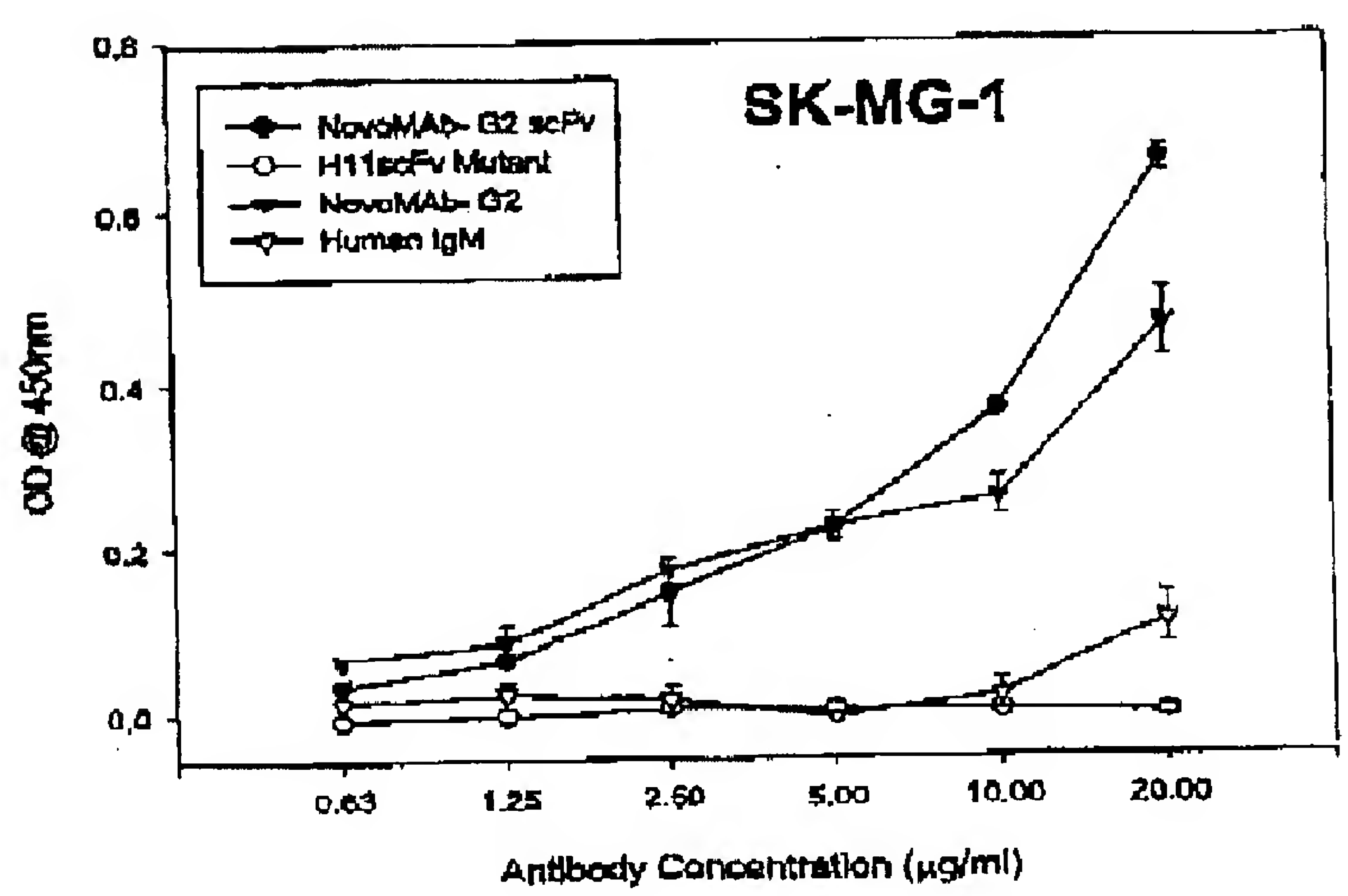

SK-MG-1
NovoMAb- G2 scFv
H11scFv Mutant
NovoMAb- G2
Human IgM
OD @ 450nm
0,8
0,6
0,4
0,2
0,0
0.63
1.25
2.50
5.00
10.00
20.00
Antibody Concentration (µg/ml)

Fig. 5

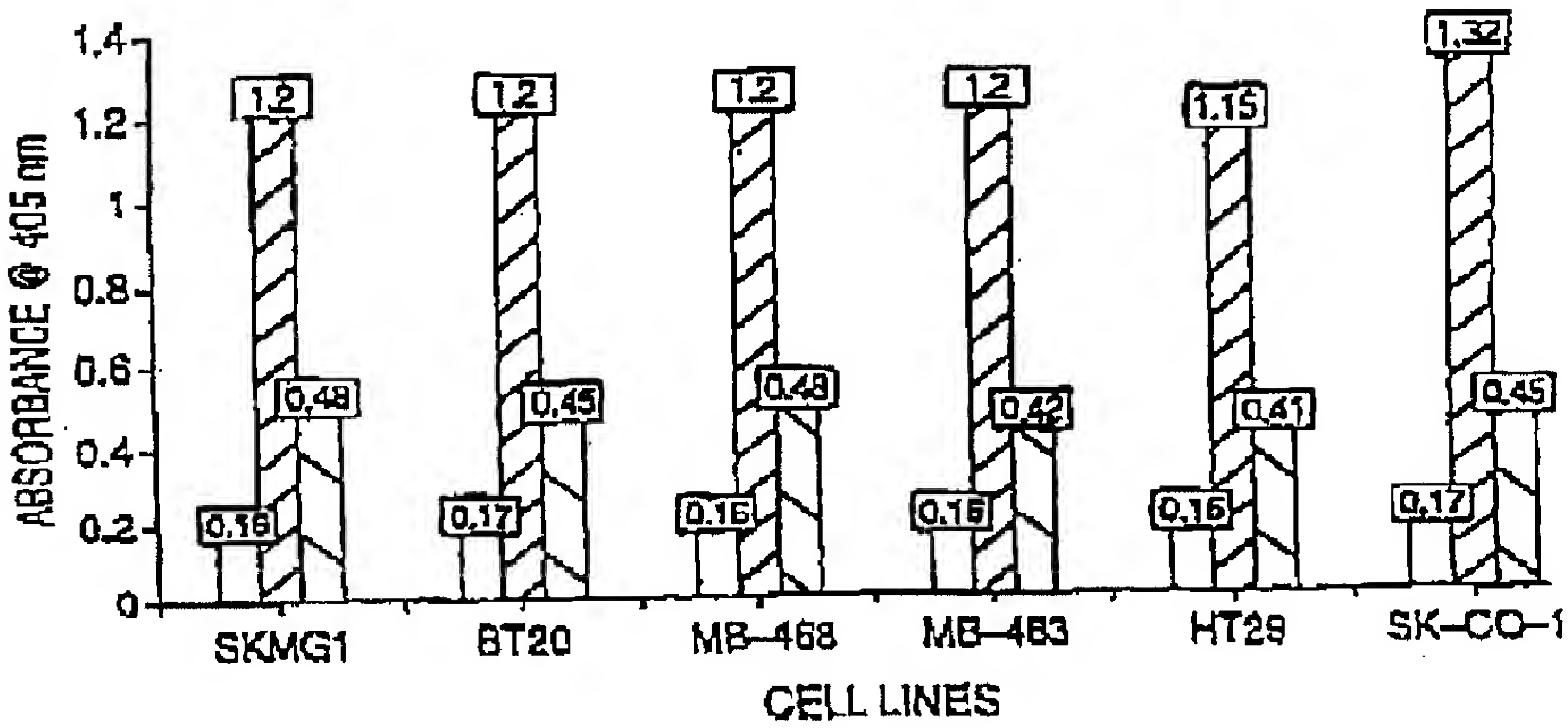

1.4
1.2
1
0.8
0.6
0.4
0.2
0
ABSORBANCE @ 405 nm
0.16
1.2
0.48
0.17
1.2
0.45
0.16
1.2
0.48
0.16
1.2
0.42
0.16
1.15
0.41
0.17
1.32
0.45
SKMG1
BT20
MB-468
MB-483
HT29
SK-CO-1
CELL LINES

Figure 6

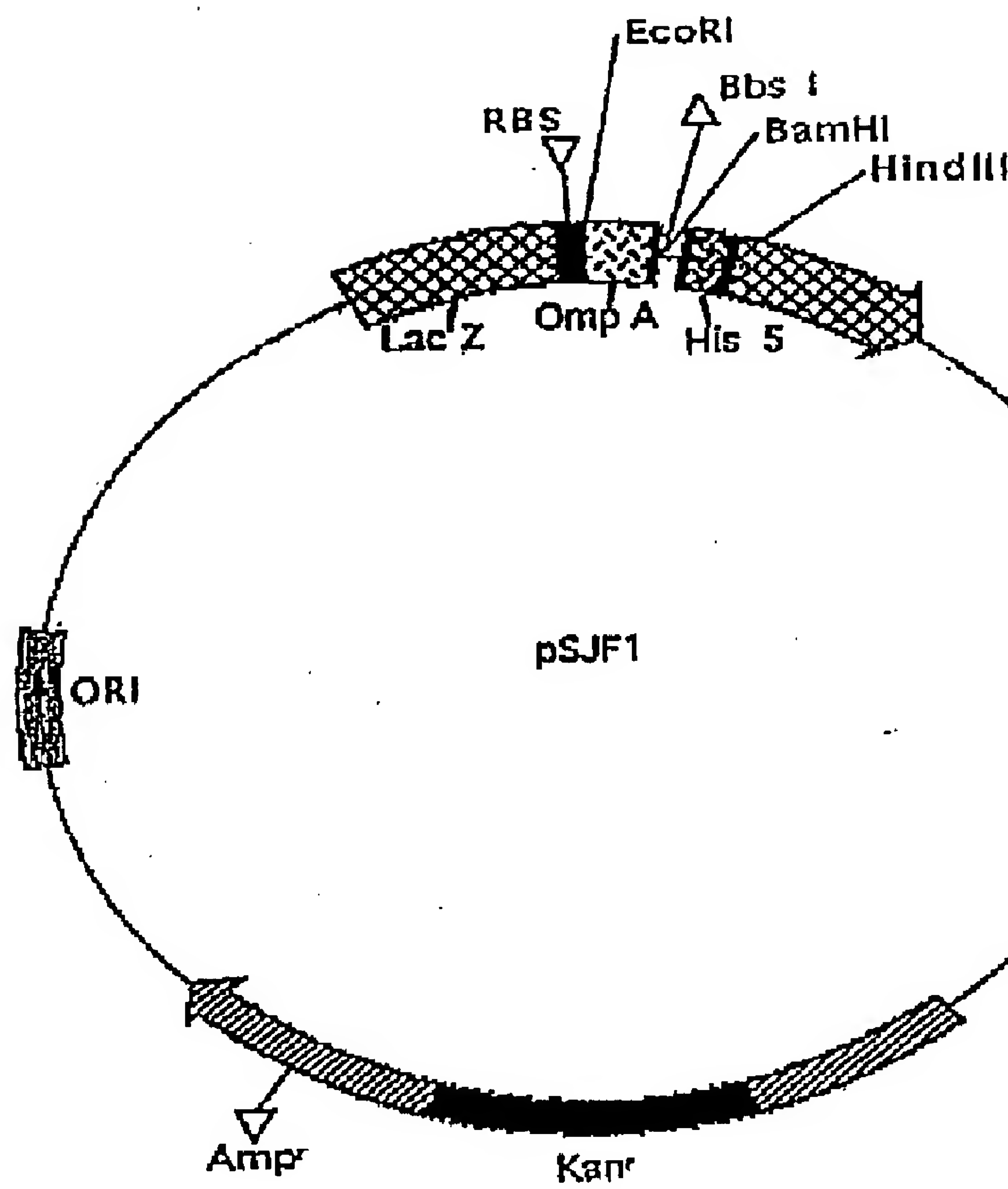

EcoRI
Bbs I
RBS
BamHI
HindIII
Lac Z
Omp A
His 5
pSJF1
ORI
Amp^r
Kan^r

Figure 7A. Effect of H11 scFv-TNf and H11 scFv alone on the growth of GI-105, a human melanoma

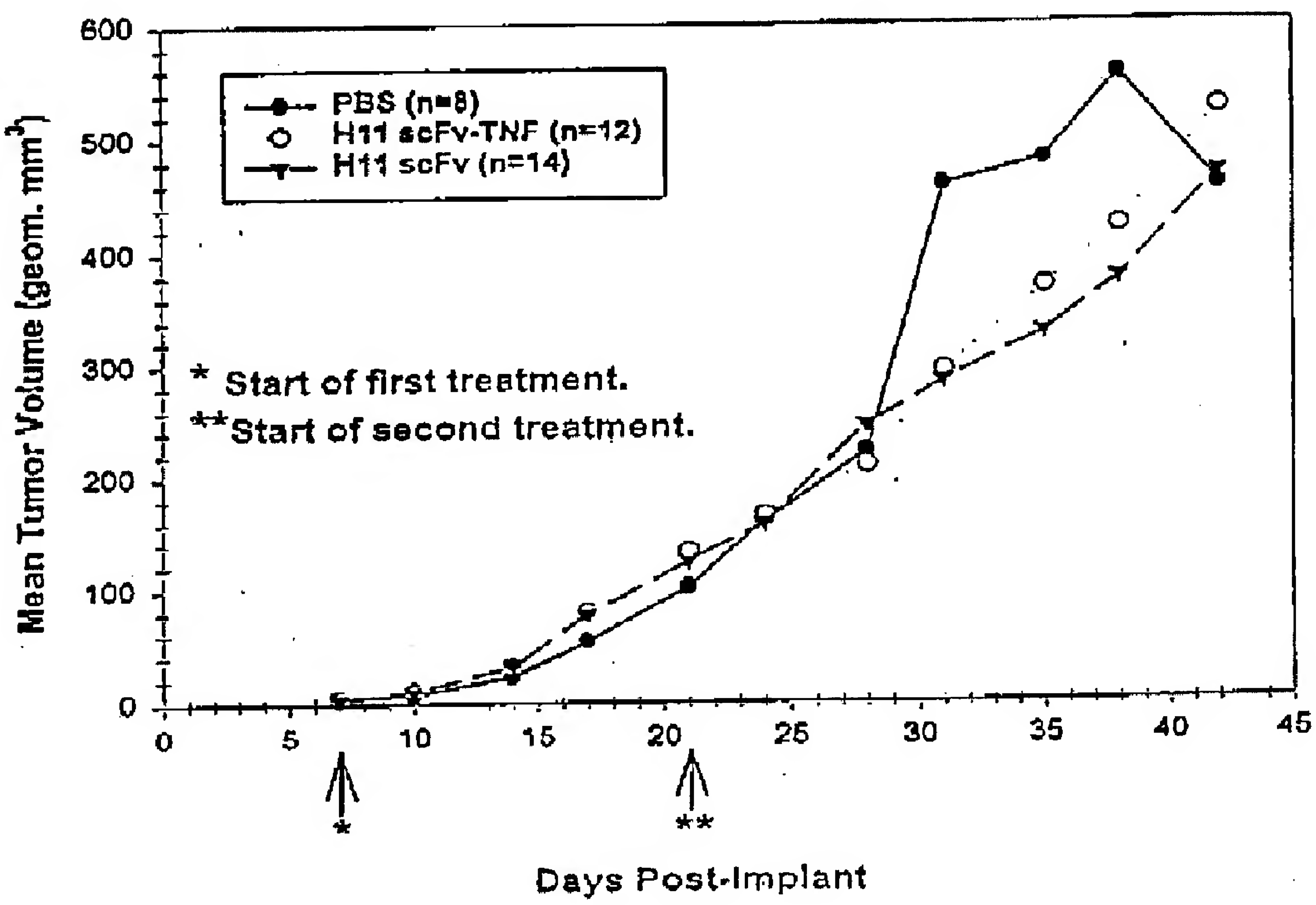


Figure 7B. Effect of H11 scFv-TNF and H11 scFv on the growth of Daudi, a human lymphoma

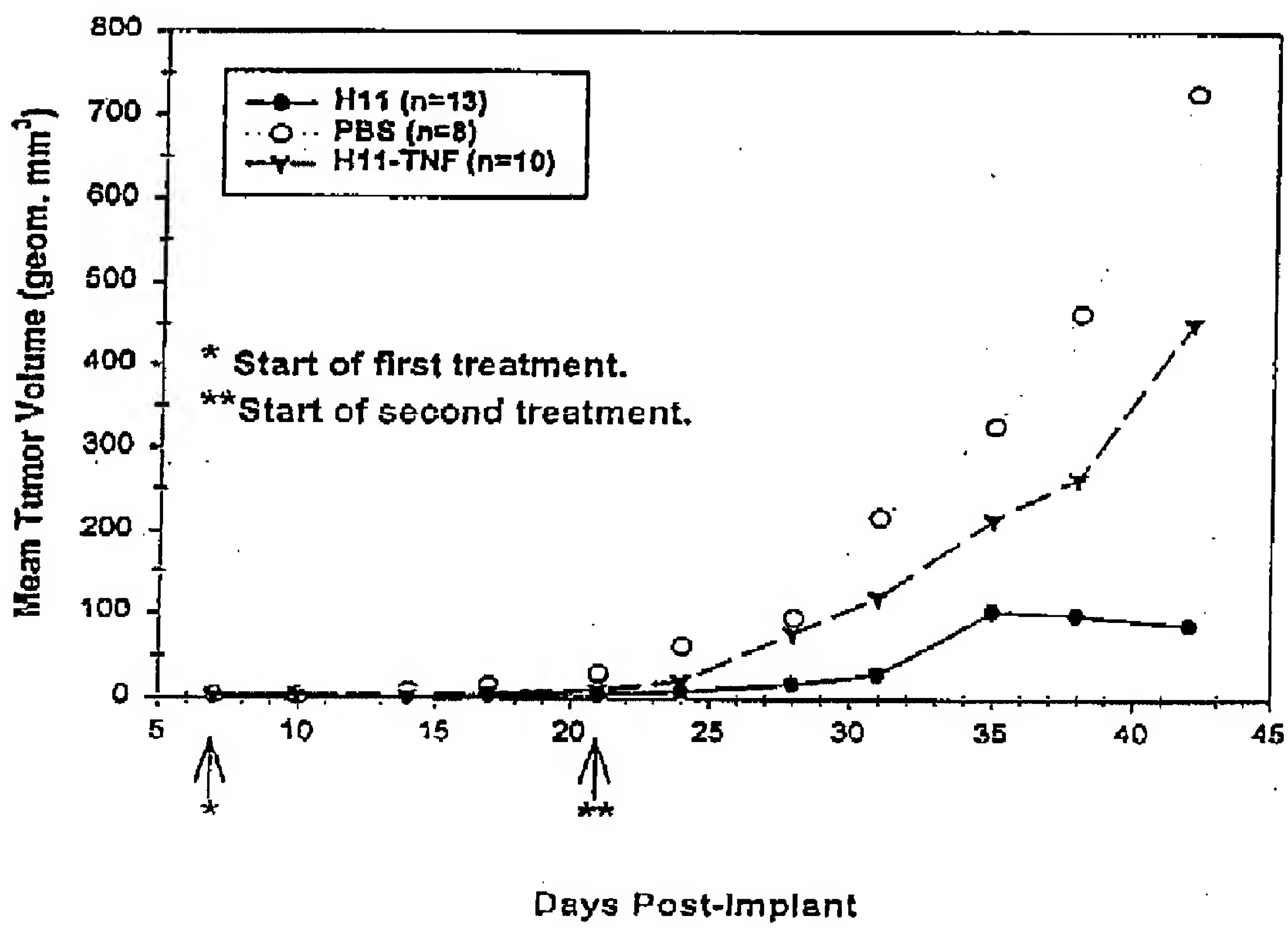


Figure 7C. The effect of H11 scFv and H11 scFv-TNF on the growth of GI-101 a human breast adenocarcinoma